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EXPERIMENTAL ARTICLES

Initial Stages of 2,4,6-Trinitrotoluene Transformation by Microorganisms

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Abstract—Screening of a wide range of microorganisms (32 strains) isolated from various anthropogenic and natural environments and of a number of collection strains showed that the early stages of 2,4,6-trinitrotoluene (TNT) transformation by the majority of the strains studied resulted in the formation of hydroxylaminodinitrotoluenes (HADNTs). The levels of HADNTs were in a number of cases comparable to the initial TNT level. The alternative reductive attack on TNT through the reduction of the aromatic ring was not characteristic of most of the prokaryotes studied. The susceptibility to the toxic effect of TNT was different for gram-positive and gram-negative bacteria.

Key words: 2,4,6-trinitrotoluene, initial transformation, hydroxylaminodinitrotoluenes.

One of the crucial problems of the present is protection of the environment from pollution with anthropogenic substances that accumulate in living organisms and affect metabolic processes.

One of the major groups among synthetic pollutants is comprised of nitroaromatic compounds, 2,4,6-trinitrotoluene (TNT) in particular. A great amount of TNT was produced during World War II, and the most part of it still exists. Even after 50 years after pollution, TNT is found in soils in large concentrations, up to 9600 mg/kg [1]. The slowness of the process of biological transformation indicates that TNT inhibits microbial activity.

TNT is mainly transformed through reducing reactions. According to a number of researchers, most aerobic bacteria reduce TNT to monoamino derivatives. Only strict anaerobes have been shown to reduce TNT to 2,4,6-triaminotoluene [2]. On the other hand, a series of studies revealed that some microorganisms are able to transform TNT with the formation of hydroxylaminodinitrotoluenes (HADNTs) [3, 4, 9]. An alternative way of the initial TNT transformation, namely, incomplete reduction of the aromatic ring with the formation of the Meisenheimer hydride-complex, has been described for a number of microorganisms [4–6]. Although a number of studies demonstrated the possibility of a partial TNT mineralization [7–9], the involvement of TNT in mineralization processes is insignificant both in nature and under laboratory conditions, which demonstrates the existence of metabolic barriers at the key stages of its transformation. The limited transformation of TNT by most microorganisms and insufficient knowledge of the mechanisms controlling these processes and impeding TNT mineralization and its involvement in the carbon and nitrogen cycles are problems yet to be solved. For adequate assessment of the bottlenecks of TNT transfomation, its initial stages should be studied in a wide range of microorganisms since these stages may result in the formation of early metabolites inhibiting further transformation. Hence, studies aimed at revealing crucial points that limit TNT metabolism are of considerable importance.

The aim of this work was to reveal the mechanisms of the initial attacks on TNT characteristic of various groups of prokaryotes.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. Microbial strains obtained from the collection of the Laboratory of Ecological Biotechnology and Biomonitoring at Kazan State University were isolated from different environments, such as oil-polluted soils, oil slime, and atmospheric emissions.

Strain sensitivity to TNT. For sensitivity tests, medium A of the following composition was used (g/l distilled water): glucose, 5.0; $MgSO_4$, 0.25; Na_2HPO_4, 4.5; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.0; yeast extract, 0.05. The medium was supplemented with a TNT solution in ethanol to a TNT concentration varied from 15 to 200 mg/l.

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TNT transformation by cell suspensions. Cells were grown until the late exponential phase in nutrient broth; harvested by centrifugation at 5000 *g* for 15 min; washed twice with 16 mM phosphate buffer, pH 6.0; and resuspended in the same buffer to obtain an optical density A_{600} of 1.0. TNT was added to a concentration of 0.44 mM. The incubation mixture contained 5 mM glucose as the source of reducing equivalents. The incubation was performed on a shaker (120 rpm) in 250-ml flasks containing 50 ml of incubation mixture.

TNT transformation by growing cultures. Cultures were incubated in 250-ml flasks containing 50 ml of medium A at 28°C with shaking (120 rpm). TNT was added in maximal concentrations at which growth was observed. The inoculum cultures were grown in medium A without TNT for 24 h. The initial optical density was adjusted to A_{600} = 0.02. Cell-free culture liquid was used as a control.

After the incubation, the suspensions were centrifuged and the concentrations of TNT and its derivatives were determined in the culture liquid.

Analysis of products of TNT transformation. TNT derivatives were analyzed on an LKB 2150 liquid chromatograph as described earlier [4].

RESULTS

Gram-negative bacteria were found to be more resistant to TNT. Minimal growth-preventing TNT concentrations varied from 100 to 200 mg/l for gram-negative strains (except for *Pseudomonas* sp. EN1561) and from 15 to 100 mg/l for gram-positive strains (table). It merits attention that certain gram-positive strains isolated by us were more resistant to TNT than most of those described earlier [10–13].

Since resting cells are more resistant to TNT than growing cultures, the initial TNT concentration was adjusted to 0.44 mM in study of TNT transformation by cell suspensions. During incubation (5 h), most gram-negative strains transformed 90–100% of TNT (table), whereas gram-positive strains transformed only 50–80% of TNT.

Figure 1 presents the dynamics of TNT transformation by cell suspensions and accumulation of major early metabolites for a number of strains (gram-positive and gram-negative strains that accumulated maximal or minimal quantities of HADNTs, respectively).

We performed HPLC analysis of the incubation mixtures after the cells had been removed, which allowed us to reveal the patterns of the initial TNT transformation. It is most significant that TNT transformation by the majority of microorganisms was limited to the formation of HADNTs as the major products (table), whereas the level of compounds with at least one completely reduced nitro group (ADNT) did not exceed 10% of the initial TNT concentration and in most cases comprised 0–3%.

Two of the strains studied (*Pseudomonas* sp. ZS50 and *P. aeruginosa* ZS31) were capable of four-electron reduction of the second nitro group with the formation of dihydroxylaminonitrotoluenes. Although we did not reveal this capability in other strains, the possibility of it cannot be rejected since dihydroxylaminonitrotoluenes are highly unstable in water solutions [14] and could transform into other compounds during the procedure of their identification.

It should be noted that HADNTs persisted in incubation mixtures and culture liquids for rather prolonged periods of time (more than 100 h). However, Wang *et al.* [14] showed that HADNTs are unstable in water solutions in the presence of molecular oxygen and are reduced into nitroso derivatives, which form dimers (2,2'-azoxy and 4,4'-azoxy derivatives) with the initial HADNTs. Indeed, in our experiments, HADNTs were unstable in deionized water but much more stable in incubation solutions and culture liquids, in which their concentration did not change during 9 h of incubation (Fig. 2). This might result from the presence of biological substances that prevent HADNT oxidation in incubation/culture liquids.

Taking into consideration the patterns of TNT transformation by cell suspensions, we found it reasonable to study TNT transformation in growing cultures. The strains were grown in medium A containing TNT in maximal concentrations at which growth was observed. We found that the initial TNT transformation by growing cultures also resulted in the formation of isomeric HADNTs, relative HADNT levels being 1.3–2.3 times lower than those in cell suspensions (Fig. 3). Subsequently, only a minor proportion of HADNTs was reduced to monoamino derivatives, whereas the major proportion transformed into unidentified products.

On the other hand, growing cultures performed more profound reductive transformation of TNT. The levels of monoamino derivatives in growing cultures amounted to 0.05–0.15 mM, being 7–26 times higher than the levels in cell suspensions. Although TNT was transformed into isomeric monoamino derivatives (considering the identified products of transformation), the total level of these compounds was in most cases far lower than the initial HADNT level.

DISCUSSION

Lately, some works were published that reported on the microbial metabolic potential of various microorganisms for use in degradation and detoxication of TNT and its stable derivatives [4, 8, 10].

It merits attention that the type of cell wall, the susceptibility to TNT, and the degree of its transformation are interrelated. Thus, we found that gram-positive bacteria and yeasts were more susceptible to TNT than gram-negative bacteria, and no gram-positive strain was capable of complete transformation of 0.44 mM TNT. Our data are in accordance with the previously

Table

Strains	Source*	TNT susceptibility, mg/l**	TNT transformed, %***	Metabolites, mM		
				HADNTs	ADNT	H-TNT
Gram-positive bacteria						
Bacillus sp. ZS19 (Figs. 1, 3a)	1	50	80	0.08	0	0.04
Bacillus cereus ZS18	1	85	84	0.13	0	0.03
Sarcina sp. IC1	2	100	86	0.25	0.04	0
Sarcina sp. IC2 (Figs. 1, 3b)	2	100	80	0.31	0.01	0
EN14	1	30	84	0.17	0.02	0.02
EN17	1	50	70	0.18	0.02	0.04
EN1201	1	50	84	0.14	0.05	0
EN811	1	25	86	0.25	0.007	0
Bacillus subtilis JH642	3	15	70	0.04	0.02	0
B. intermedius 7P	3	25	65	0.10	0.003	0
B. circulans BCF-247	3	15	70	0.11	0	0
B. thuringiensis var. *subtoxicus*	3	35	80	0.13	0.01	0
B. intermedius 10-41	3	15	76	0.13	0.003	0
Gram-negative bacteria						
Pseudomonas aeruginosa ZS31	3	150	100	0.36	0	0
Pseudomonas fluorescens ZS32	1	200	100	0.37	0.009	0
Pseudomonas putida ZS41	1	100	100	0.38	0.004	0
Pseudomonas sp. ZS50	1	150	100	0.20	0	0.08
Pseudomonas putida ZS61	3	200	100	0.29	0.009	0
Pseudomonas putida ZS71	1	200	100	0.27	0.009	0
Pseudomonas sp. ZS81	1	150	100	0.35	0	0.06
Pseudomonas fluorescens ZS82	3	150	100	0.35	0.004	0
Pseudomonas sp. EN1582	1	200	100	0.24	0	0
Pseudomonas sp. EN1561	4	50	50	0.13	0	0
ZS10	4	100	100	0.35	0	0.08
ZS20 (Figs. 1, 3d)	4	150	100	0.41	0	0.02
ZS42	1	125	100	0.40	0	0.03
ZS62	1	150	100	0.29	0.004	0.04
ZS72	1	150	100	0.27	0.004	0
ZS180 (Figs. 1, 3c)	1	150	100	0.16	0	0
EN21	1	150	86	0.23	0.004	0
EN22	1	100	98	0.29	0.008	0
EN1181	1	100	84	0.31	0.021	0

* Sources of isolation: 1, oil slime; 2, atmospheric emissions of a petrochemical plant; 3, the collection of Kazan State University; 4, soil.

** Maximal TNT concentration (mg/l) in a medium at which growth was observed.

*** Percent of the initial concentration (0.44 mM).

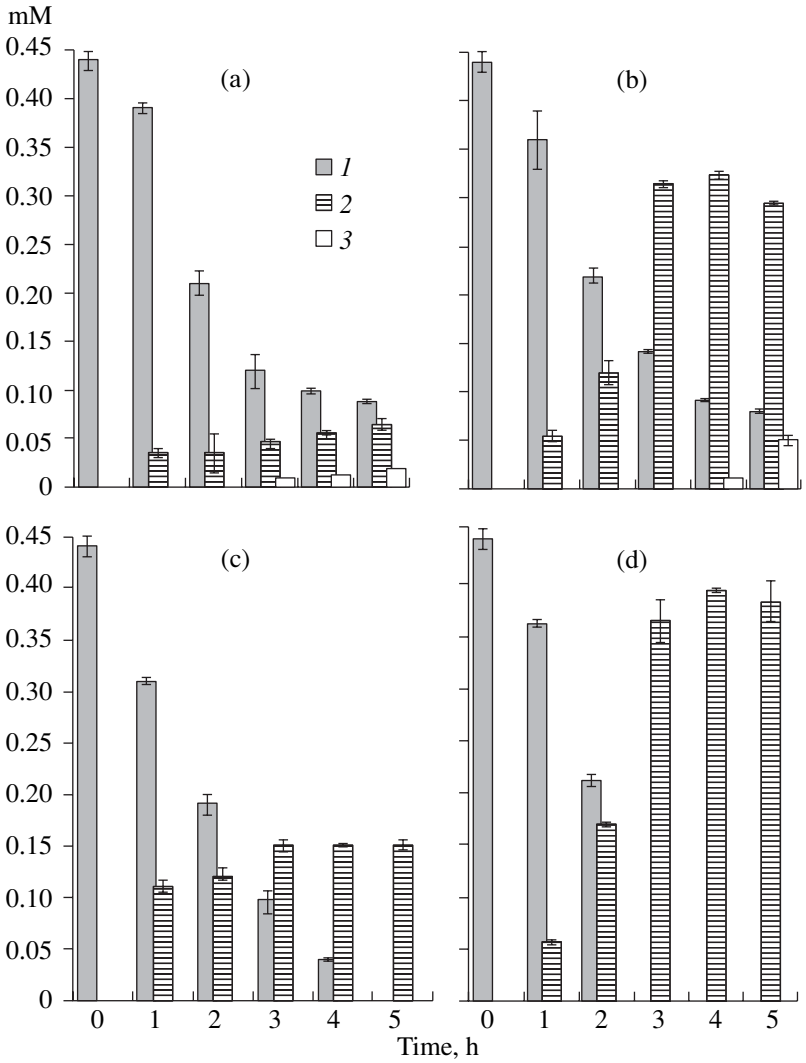

Fig. 1. Dynamics of TNT transformation by cell suspensions of the gram-positive bacteria (a) *Bacillus* sp. ZS19 and (b) *Sarcina* sp. IC2 and the gram-negative bacteria (c) ZS180 and (d) ZS20: (*1*) TNT, (*2*) HADNTs, and (*3*) 3-monoaminodinitrotoluenes.

offered hypothesis that aerobic gram-positive bacteria are more susceptible to the xenobiotic and less active in its transformation [10].

It is of fundamental importance that the attacks of most strains on TNT were limited and resulted in the formation of isomeric HADNTs as major products of transformation (table).

The possibility of HADNT formation was first shown for the micromycete *Phanerochaete chrysosporium* [9]. However, the maximal HADNT concentration detected by the authors was approximately 40 μM, while the initial TNT concentration was 100 mM. Later, the study of fungal species as potential TNT destructors revealed that only a few strains of 91 studied produced HADNTs and only as by-products and in minor quantities [8]. Kim and Song [15] characterized isomeric hydroxylamino derivatives as the earliest detectable products of TNT transformation by seven micromycete strains. No data on HADNT formation were reported in the paper on the comparative study of TNT transformation by gram-positive and gram-negative microorganisms [10]. Earlier, we demonstrated massive HADNT production for two lactobacilli [3]

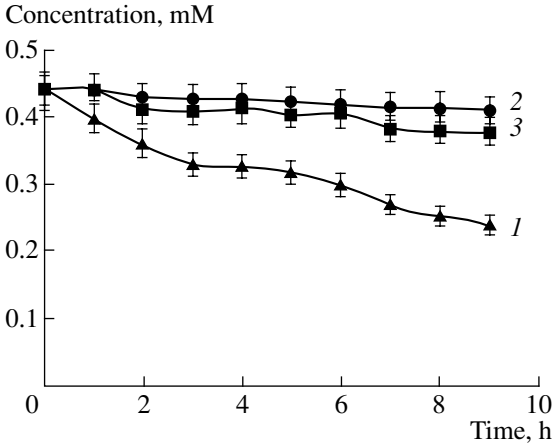

Fig. 2. HADNT stability in (*1*) deionized water, (*2*) culture liquid, and (*3*) incubation mixture.

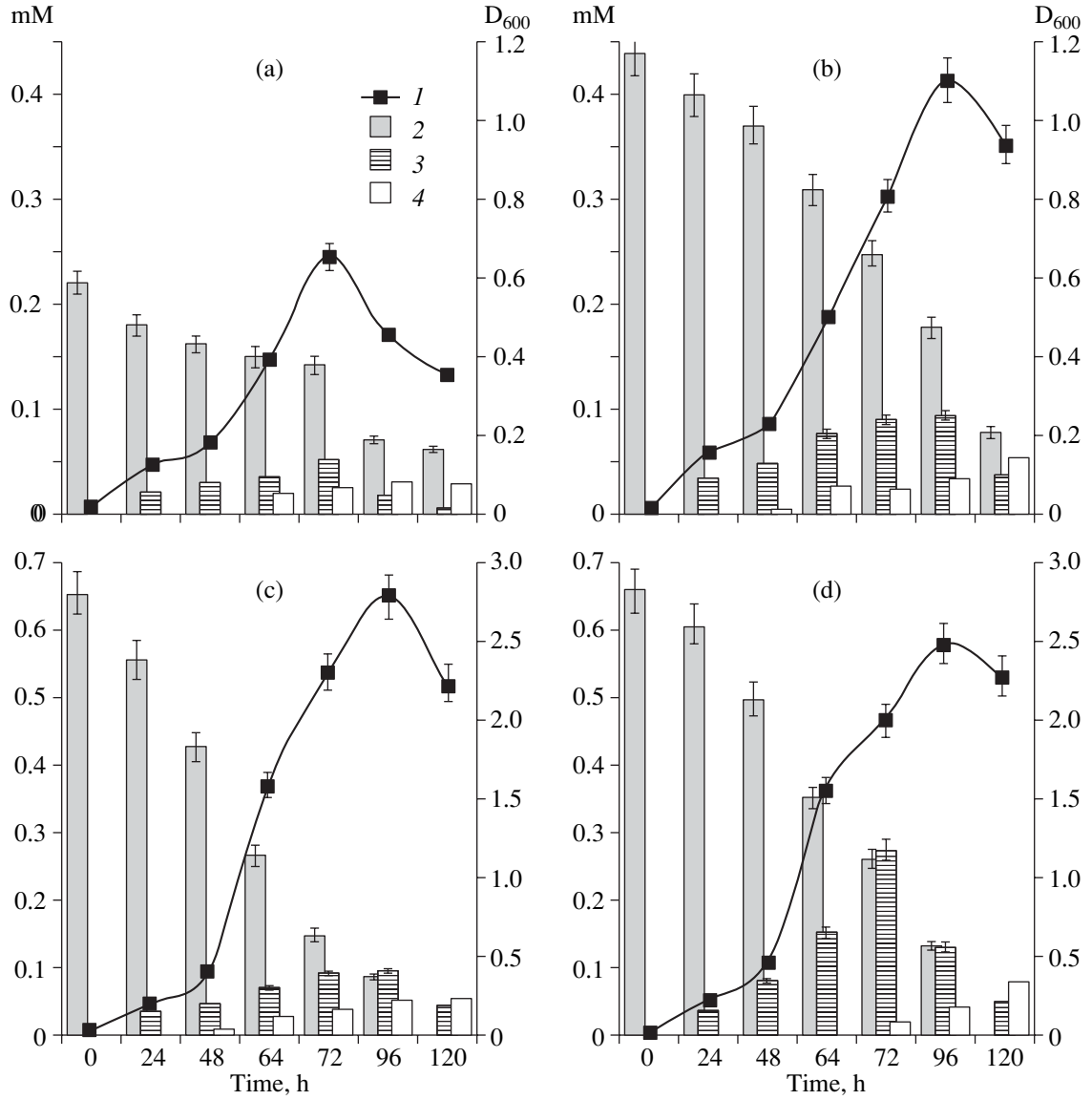

Fig. 3. Dynamics of TNT transformation by growing cultures of the gram-positive bacteria (a) *Bacillus* sp. ZS19 and (b) *Sarcina* sp. IC2 and the gram-negative bacteria (c) ZS180 and (d) ZS20: (*1*) growth, (*2*) TNT, (*3*) HADNTs, and (*4*) monoaminodinitrotoluenes.

and one yeast [4]. These strains performed stoichiometric TNT reduction with the formation of monohydroxylamino derivatives up to concentrations of 95–100% from the initial TNT level.

Most microorganisms of different metabolic groups are known to produce mono and diamino derivatives as the end detectable products of aerobic TNT transformation [9]. We found that growing cultures transformed TNT mainly into hydroxylamino derivatives, although monoaminodinitrotoluenes were produced as well (Fig. 3).

Limited TNT transformation by cell suspensions may possibly be determined by the blocking effect of HADNTs on certain physiological functions of microbial cells, particularly, regeneration of reductive cofactors required for TNT reduction. Earlier, we demonstrated that the key oxidoreductases of lactobacilli were almost entirely inhibited by HADNTs [3].

As to the partial reduction of the aromatic ring described in our previous works for yeasts [4], most of the bacterial strains studied were incapable of such reduction.

TNT transformation with the formation of HADNTs as major metabolites is of particular interest since HADNT toxicity and mutagenicity are much higher that those of the initial xenobiotic [4]. For a number of nitroaromatic compounds, including TNT, the formation of nitroso and hydroxylamino derivatives provides their metabolic activation, resulting in the

formation of adducts with biological macromolecules, such as proteins and nucleic acids [16, 17].

As the strains used in this study were isolated from the surrounding environment (air, soils), there is a risk of human poisoning with the toxic and mutagenic intermediates of TNT reduction.

Thus, we found that the majority of microorganisms performed the initial TNT transformation with the formation of various levels of isomeric HADNTs. Incomplete TNT reduction without further massive and rapid transformation of intermediates through the usual pathways of nitro group reduction may indicate the existence of metabolic barriers.

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REFERENCES

1. George, S.E., Huggins-Clark, G., and Brooks, L.R., Use of a *Salmonella* Microsuspension Bioassay to Detect the Mutagenicity of Munitions Compounds at Low Concentrations, *Mutat. Res.*, 2001, vol. 490, no. 1, pp. 45–56.
2. Spain, J., *Biodegradation of Nitroaromatic Compounds and Explosives*, London: CRC, 2000.
3. Naumov, A.V., Suvorova, E.S., Boronin, A.M., Zaripova, S.K., and Naumova, R.P., Transformation of 2,4,6-Trinitrotoluene into Toxic Hydroxylamino Derivatives by Lactobacilli, *Mikrobiologiya*, 1999, vol. 68, no. 1, pp. 65–71.
4. Zaripov, S.A., Naumov, A.V., Nikitina, E.V., and Naumova, R.P., Alternative Pathways of the Initial Transformation of 2,4,6-Trinitrotoluene by Yeasts, *Mikrobiologiya*, 2002, vol. 71, no. 5, pp. 648–653.
5. Vorbeck, C., Lenke, H., Fischer, P., Spain, J., and Knackmuss, H.-J., Initial Reductive Reactions in Aerobic Microbial Metabolism of 2,4,6-Trinitrotoluene, *Appl. Environ. Microbiol.,* 1998, vol. 64, no. 1, pp. 246–252.
6. French, P.D., Nicklin, S., and Bruce, N.C., Aerobic Degradation of 2,4,6-Trinitrotoluene by *Enterobacter cloacae* PB2 and by Pentaerythritol Tetranitrate Reductase, *Appl. Environ. Microbiol.*, 1998, vol. 64, no. 8, pp. 2864–2868.
7. Funk, S., Roberts, D., Crawford, D., and Crawford, R., Initial-Phase Optimization for Bioremediation of Munition Compound–Contaminated Soils, *Appl. Environ. Microbiol.*, 1993, vol. 59, no. 7, pp. 2171–2177.
8. Scheibner, K., Hofrichter, M., Herre, A., Michels, J., and Fritsche, W., Screening for Fungi Intensively Mineralizing 2,4,6-Trinitrotoluene, *Appl. Microbiol. Biotechnol.*, 1997, vol. 47, pp. 452–457.
9. Michels, J. and Gottschalk, G., Inhibition of the Lignin Peroxidase of *Phanerochaete chrysosporium* by Hydroxylamino-Dinitrotoluene, an Early Intermediate in the Degradation of 2,4,6-Trinitrotoluene, *Appl. Environ. Microbiol.*, 1994, vol. 60, pp. 187–194.
10. Fuller, M.E. and Manning, J.F., Aerobic Gram-Positive and Gram-Negative Bacteria Exhibit Differential Sensitivity to and Transformation of 2,4,6-Trinitrotoluene (TNT), *Curr. Microbiol.*, 1997, vol. 35, pp. 77–83.
11. Klausmeier, R.E., Osmon, J.L., and Walls, D.R., Effect of Trinitrotoluene on Microorganisms, *Dev. Ind. Microbiol.*, 1974, vol. 15, pp. 309–317.
12. Vorbeck, C., Lenke, H., Fisher, P., and Knackmuss, H.-J., Identification of a Hydride-Meisenheimer Complex as a Metabolite of 2,4,6-Trinitrotoluene by a *Mycobacterium* Strain, *J. Bacteriol.*, 1994, vol. 176, no. 3, pp. 932–934.
13. Vanderberg, L., Perry, J., and Unkefer, P., Catabolism of 3,4,6-Trinitrotoluene by *Mycobacterium vaccae, Appl. Microbiol. Biotechnol.*, 1995, vol. 43, pp. 937–945.
14. Wang, C.-Y., Zheng, D., and Hughes, J.B., Stability of Hydroxylamino- and Aminointermediates from Reduction of 2,4,6-Trinitrotoluene, 2,4-Dinitrotoluene, and 2,6-Dinitrotoluene, *Biotechnol. Lett.*, 2000, vol. 22, pp. 15–19.
15. Kim, H.-Y. and Song, H.-G., Comparison of 2,4,6-Trinitrotoluene Degradation by Seven Strains of White Rot Fungi, *Curr. Microbiol.*, 2000, vol. 41, no. 5, pp. 0317–0320.
16. Rafii, F., Selby, A.L., Newton, R.K., and Cerniglia, C.E., Reduction and Mutagenic Activation of Nitroaromatic Compounds by a *Mycobacterium* sp., *Appl. Environ. Microbiol.*, 1994, vol. 60, no. 12, pp. 4263–4267.
17. Neumann, H.G., Albrecht, O., Vandorp, C., and Zwirnerbaier, I., Macromolecular Adducts Caused by Environmental Chemicals, *Clin. Chem.*, 1995, vol. 41, part 2, suppl. S, pp. 1835–1840.