Applied and Environmental Microbiology, Dec. 2006, p. 7945–7948 Vol. 72, No. 12
0099-2240/06/$08.00+0 doi:10.1128/AEM.01052-06

Aerobic Growth of *Escherichia coli* with 2,4,6-Trinitrotoluene (TNT) as the Sole Nitrogen Source and Evidence of TNT Denitration by Whole Cells and Cell-Free Extracts▽†

Ben Stenuit,[1] Laurent Eyers,[1] Raoul Rozenberg,[2] Jean-Louis Habib-Jiwan,[2] and Spiros N. Agathos[1]*

Unit of Bioengineering, Department of Applied Chemistry and Bioindustries,[1] and Mass Spectrometry Unit, Department of Chemistry,[2] Catholic University of Louvain, Louvain-la-Neuve, Belgium

Received 5 May 2006/Accepted 25 September 2006

***Escherichia coli* grew aerobically with 2,4,6-trinitrotoluene (TNT) as sole nitrogen source and caused TNT's partial denitration. This reaction was enhanced in nongrowing cell suspensions with 0.516 mol nitrite released per mol TNT. Cell extracts denitrated TNT in the presence of NAD(P)H. Isomers of amino-dimethyl-tetranitrobiphenyl were detected and confirmed with U-^{15}N-labeled TNT.**

2,4,6-Trinitrotoluene (TNT) is recalcitrant to microbial degradation. Denitration (defined as the release of nitrite) is a critical step for further mineralization of TNT (19). A well-described TNT denitration pathway involves a nucleophilic addition of hydride ions to the aromatic ring with subsequent nitrite release. Three enzymes performing this addition in the presence of NAD(P)H have been characterized so far: pentaerythritol tetranitrate reductase of *Enterobacter cloacae* PB2 (9), xenobiotic reductase B (XenB) of *Pseudomonas fluorescens* I-C (15), and *N*-ethylmaleimide (NEM) reductase of *Escherichia coli* (21). In vitro denitration of TNT with purified NEM reductase was described by Williams et al. (21), but the authors did not provide quantitative data with *E. coli* cells. Also, several reports have described the reduction of TNT by *E. coli* but not its denitration (6, 14, 22, 23). Only one recent study has mentioned denitration of TNT by *E. coli*, but no quantitative data were provided and TNT was not the sole nitrogen source (13). The objectives of this study were to determine the kinetics of TNT denitration by *E. coli* and identify TNT denitrated metabolites.

E. coli strains EPI300 (Epicentre Technologies, Madison, WI) and LK111 (24) were routinely cultivated at 37°C in Luria-Bertani (LB) broth. Cells were harvested at mid-exponential phase and washed three times with phosphate-buffered saline (containing, per liter, 7 g of $Na_2HPO_4 \cdot 12H_2O$, 3 g of KH_2PO_4, 1 g of NaCl). Cells were resuspended in 20 ml of phosphate-buffered saline and used for TNT biodegradation assays.

TNT was obtained from Nobel Explosives (Châtelet, Belgium) and was 99.5% pure by high-performance liquid chromatography. Growing cell experiments were carried out in modified mineral salts medium (10) containing 20 mM of glycerol or glucose and TNT as the sole nitrogen source. The medium was inoculated at an optical density at 600 nm (OD_{600}) of 0.025 and incubated at 37°C and 250 rpm. Controls consisted of flasks without TNT, flasks without cells, flasks without a carbon source, and flasks with 200 ppm of Hg_2Cl_2 and without a carbon source. Nitrite, TNT, and metabolites were quantitatively determined as previously described (7).

Bacterial growth of *E. coli* EPI300 was observed with glycerol and TNT (606 μM on the basis of high-performance liquid chromatography analysis) as the sole nitrogen source (Fig. 1A). The growth was relatively fast over the first 26 h, reaching a plateau of 0.120 OD_{600} units after 117 h of incubation. With glucose and TNT (588 μM), the bacterial growth profile was similar but the OD_{600} reached 0.320 after 117 h (Fig. 1B). Without TNT, no significant increase of the OD_{600} was observed (data not shown). The increase in OD_{600} correlated with a decrease in TNT concentration. With glycerol and TNT, the residual concentration of the latter after 117 h was 392.5 μM. Concomitant with this partial TNT removal, a gradual accumulation of TNT reduced metabolites was observed (Fig. 1A), including 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT), 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (2,2′-azoxy), and 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (4,4′-azoxy). The concentration of 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT) was not significant. With glucose, almost all the initial TNT had disappeared by 26 h (Fig. 1B). A gradual accumulation of 4-A-2,6-DNT, 4,4′-azoxy, and 2,2′-azoxy was observed (Fig. 1B). The concentration of 2-A-4,6-DNT was not significant.

In addition to bacterial growth, we observed a release of nitrite (Fig. 1C). With glycerol and TNT, the concentration of nitrite increased during the first 26 h to a level of 76 μM and evolved slowly upon further incubation to 100 μM after 117 h (i.e., 0.165 mol of nitrite released/mol of TNT added or 0.468 mol of nitrite released/mol of TNT transformed) (Fig. 1C). With glucose and TNT, 48 μM of nitrite was measured after 26 h and its concentration remained relatively constant thereafter (42 μM after 117 h of incubation, i.e., 0.071 mol of nitrite released/mol of TNT added) (Fig. 1C). The nitrite detected in both experiments resulted unambiguously from a TNT denitration metabolism, given the complete absence of nitrite release in control flasks (data not shown).

We investigated whether another strain of *E. coli* (i.e., *E. coli* LK111) was able to use TNT as the sole nitrogen source

* Corresponding author. Mailing address: Unit of Bioengineering, Place Croix du Sud 2/19, 1348 Louvain-la-Neuve, Belgium. Phone: 32 10 47 36 44. Fax: 32 10 47 30 62. E-mail: spiros.agathos@uclouvain.be.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 29 September 2006.

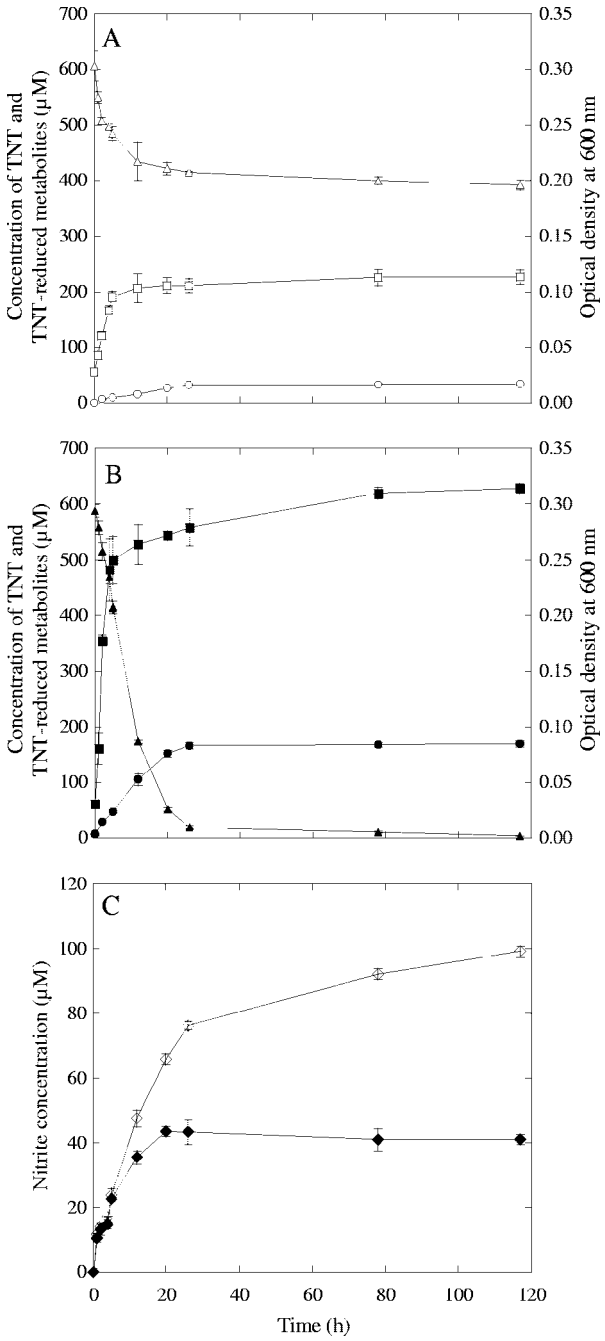

FIG. 1. Growing cell experiments with *E. coli* EPI300. Results shown are for glycerol (20 mM) as the carbon source and TNT (606 μM) as the sole nitrogen source (open symbols) or glucose (20 mM) as the carbon source and TNT (588 μM) as the sole nitrogen source (closed symbols). The time course of cell growth (squares, right axis), TNT removal (triangles, left axis), and production of TNT reduced metabolites (2-A-4,6-DNT, 4-A-2,6-DNT, 2,2′-azoxy, and 4,4′-azoxy) (circles, left axis) (A and B) and nitrite release (lozenges) (C) are shown. Error bars represent the standard deviations of triplicate cultures.

and denitrate it. With glycerol and TNT (586 μM), the extent of bacterial growth was similar to that observed with *E. coli* EPI300 (data not shown). In addition, nitrite accumulated in the culture medium (42 and 49 μM [i.e., 0.083 mol of nitrite released/mol of TNT added] after 26 and 140 h, respectively).

The use of nitrite by both strains as the sole nitrogen source was also investigated at concentrations between 50 and 2,000 μM in the presence of glycerol or glucose. The concentration of nitrite remained stable, and no growth was observed (results not shown). This was consistent with previous results (17) and explained why nitrite accumulated in the experiment shown in Fig. 1C. Since *E. coli* is unable to assimilate nitrate or nitrite under aerobic conditions (12), the use of nitrogen from TNT occurred via a metabolic pathway other than the direct release of nitrite. Recent reports have postulated the partial reduction of one or more nitro groups of TNT to its hydroxylamino moiety and the subsequent release of ammonium from the aromatic ring (3–5, 16), probably through a Bamberger-like rearrangement (11). Next, ammonium was likely used as a nitrogen source by *E. coli* via the glutamine synthetase/glutamate synthase pathway (16). The formation of 4-hydroxylamino-2,6-dinitrotoluene as a major intermediate was observed in the enzymatic reduction of TNT with extracts from *E. coli* (20) and with several enzymes of *E. coli* (21, 22). We observed a greater bacterial growth in the presence of glucose, and we found that more 4-A-2,6-DNT was produced than in the presence of glycerol (Table 1). Since 4-A-2,6-DNT was produced via successive reductions of the nitro group to nitroso and hydroxylamino intermediates (19), the latter could provide ammonium, as previously suggested (16).

Denitration of TNT was also investigated under nongrowing conditions. A 150-ml aliquot of mineral salts medium was inoculated to an OD_{600} of 1.5 with a washed *E. coli* EPI300 cell pellet obtained from an LB culture. The medium contained TNT (271 μM), and no carbon source was added. Controls included flasks without TNT and flasks without cell suspensions. TNT disappearance occurred concomitantly with the release of a significant concentration of nitrite (Fig. 2). Indeed, 120 μM of nitrite was released after 83 h, reaching a value of 140 μM after 275 h (i.e., 0.516 mol of nitrite released/mol of TNT added) (Fig. 2). In parallel, the TNT concentration decreased to 100 μM by the end of the incubation, with minimal production of TNT reduced metabolites. Taking into account the residual TNT, we calculated a TNT denitration rate of 0.818 mol of nitrite released/mol of TNT transformed. No significant nitrite release was observed in control flasks without TNT (Fig. 2) or without cells (data not shown). This confirmed that nitrite was released from TNT through biotic removal of the nitro group. In addition, our findings suggested two distinct TNT catabolic pathways: a denitration pathway favored under nongrowing conditions and a nitro reduction pathway favored under growing conditions in the presence of glucose.

The percentage of N-containing compounds produced from TNT was calculated (Table 1). Under growing conditions with glycerol, we calculated an N-mass balance of 74.9% distributed as residual TNT (64.7%) and TNT reduced metabolites (10.2%). Thus, 25.1% was missing in the balance and probably represented denitrated compounds produced (we measured 0.165 mol of nitrite released/mol of TNT added) and TNT to sustain bacterial growth. Under growing conditions with glucose, 58.5% consisted of TNT reduced metabolites (no residual TNT). Therefore, the fate of 41.5% of the initial TNT remained unknown. It likely included denitrated compounds

TABLE 1. Mass balance of TNT and TNT reduced metabolites and TNT denitration rates with *E. coli* EPI300 under different conditions

Biological system	Residual TNT (%)[a]	TNT reduced metabolites (% molar equivalent of initial TNT)[b]	TNT denitration rate based on:	
			Mol of nitrite released/mol of TNT added	Mol of nitrite released/mol of TNT transformed
Growing cells with TNT and glycerol (OD_{600}, 0.025–0.120)	64.7	10.2	0.165	0.468
Growing cells with TNT and glucose (OD_{600}, 0.025–0.320)	0.0	58.5	0.071	0.071
Resting cells with TNT and no carbon source	36.9	8.5	0.516	0.818

[a] Residual TNT (%) = $([TNT_f]/[TNT_i]) \times 100$, where $[TNT_f]$ is the final concentration of TNT and $[TNT_i]$ is the initial concentration.

[b] TNT reduced metabolites (%) = $([4\text{-A-}2,6\text{-DNT}_f]/[TNT_i]) + \{([4,4'\text{-azoxy}_f]/[TNT_i]) \times 2\} + \{([2,2'\text{-azoxy}_f]/[TNT_i]) \times 2\}, \times 100$; this equation takes into account that two molar equivalents of TNT are needed to form one molar equivalent of the azoxy compounds. The subscript *f* indicates a final concentration, and the subscript *i* indicates an initial concentration. Final concentrations were measured at the end of the incubation (117 and 275 h for growing and resting cell experiments, respectively).

(we detected 0.0714 mol of nitrite released/mol of TNT added) and TNT to sustain bacterial growth. In the resting cell experiments, 45.4% was distributed as residual TNT (36.9%) and TNT reduced metabolites (8.5%). Thus, 54.6% was missing in the balance, and it probably included denitrated compounds (we measured 0.516 mol of nitrite released/mol of TNT added).

For cell extract experiments, *E. coli* strain EPI300 was grown in mineral salts medium supplemented with TNT (600 μM) as the sole nitrogen source and glycerol (40 mM) or glucose (20 mM) as the carbon source. Another cell extract was prepared from LB-grown cells in the absence of TNT. Cells were harvested at mid-exponential phase, washed, and resuspended in 50 mM phosphate buffer (pH 7.0). Cell extracts were prepared by sonication/lysozyme treatment (18). Assay mixtures contained 440 μM TNT (with or without addition of 1 mM NADH or 1 mM NADPH), 20 mM of phosphate buffer (pH 7.0), and 1 ml of cell extracts (0.06 to 0.12 mg protein/ml) (2) to a final volume of 2 ml. Previous experiments indicated that lysozyme had no TNT denitration activity (data not shown). Mass spectrometry analyses were carried out as previously described (8) under the following conditions: vaporizer temperature, 360°C; sheath gas pressure, 20 lb/in^2; capillary temperature, 270°C; collision energy, 25 (arbitrary units).

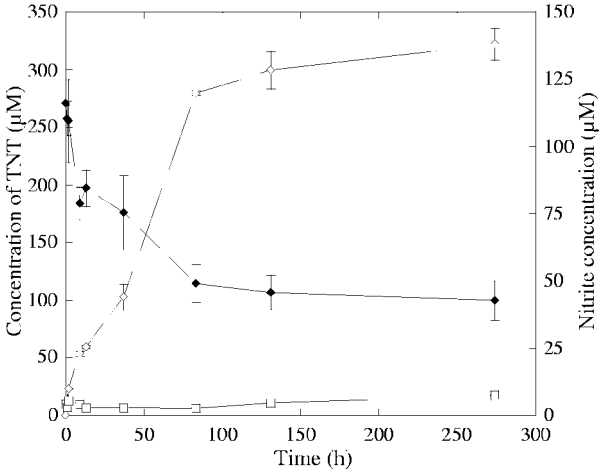

FIG. 2. Resting cell experiments with *E. coli* EPI300. Time course of TNT removal (closed lozenges) and release of nitrite (open lozenges) in the presence of TNT (271 μM) are shown. Open squares denote the time course of nitrite release in the absence of TNT. Error bars represent the standard deviations of triplicate cultures.

In the presence of NAD(P)H, denitration of TNT was observed with cell extracts (Table 2). No denitration was observed in the absence of TNT or cell extracts (data not shown). After a 19-h incubation period, TNT denitration levels with cell extracts from glycerol-grown cells reached 0.068 mol of nitrite released/mol of TNT added with NADPH and 0.093 mol of nitrite released/mol of TNT added with NADH. TNT denitration levels with extracts from glucose-grown cells were lower (i.e., 0.050 mol of nitrite released/mol of TNT added with NADPH and 0.062 mol of nitrite released/mol of TNT added with NADH). TNT denitration with extracts from LB-grown cells amounted to 0.009 mol per mol of TNT added (NADH as cofactor). In the absence of cofactors, the release of nitrite was much lower. Taken together, these results suggested the involvement of specific enzymes that may be inducible by TNT.

Mass analyses were carried out on cell extract samples from glycerol-grown cells on account of the TNT denitration rates observed. In addition to previously described TNT reduced metabolites, we detected three metabolites with higher retention times (see Fig. S1 in the supplemental material) than TNT (32.50 min). These metabolites yielded a deprotonated molecular mass ion $[M\text{-}H]^-$ at 376 Da (molecular mass, 377 Da). An *m/z* of 376 was consistent with the deprotonated molecular mass ion of a compound with an odd number of nitrogen atoms (nitrogen rule). In a previous study, Pak and colleagues (15) assigned the chemical structures of the deprotonated molecu-

TABLE 2. Nitrite release from TNT with cell extracts[a]

Cofactor	Nitrite release (mol of nitrite released/mol of TNT added)	
	Glycerol-grown cells	Glucose-grown cells
NADPH (1 mM)	30.3 ± 1.7	22.1 ± 3.4
NADH (1 mM)	40.8 ± 3.6	27.3 ± 2.6
None	6.4 ± 2.3	5.3 ± 1.9

[a] Nitrite release (mol of nitrite released/mol of TNT added) was measured after a 19-h incubation (at 37°C with agitation) of cell extracts with 440 μM TNT in a phosphate buffer (20 mM, pH 7.0). The final concentration of protein corresponded to 30 μg of protein/ml (cell extracts from glycerol-grown cells) or 60 μg of protein/ml (cell extracts from glucose-grown cells). Values shown are averages and standard deviations of triplicate experiments. *E. coli* EPI300 cells were precultured in a minimal medium with 600 μM of TNT as the sole nitrogen source. Glycerol (40 mM) or glucose (20 mM) was the carbon source.

lar mass ions $[M-H]^-$ at 376 Da to various isomers of amino-dimethyl-tetranitrobiphenyl (see Fig. S1). They postulated that these isomers were dimers resulting from the reaction between products of aromatic ring reduction and nitro group reduction (15). The significant peak of oxygen isotope observed by mass spectrometry analysis confirmed the possible formation of such dimers containing eight oxygen atoms (see Fig. S1). The deprotonated molecular mass ion at 376 Da was fragmented (see Fig. S1), and two peaks at *m*/*z* 181 and 196 represented the fragmentation of the putative dimer (see Fig. S1). When U-^{15}N-labeled TNT was used, the deprotonated molecular mass ion at 376 Da shifted to 381 Da (see Fig. S2 in the supplemental material), indicating that the dimer contained five nitrogen atoms. The peaks at *m*/*z* 183 and 199 represented the fragmentation of the ^{15}N-labeled dimer, confirming the presence of five nitrogen atoms (see Fig. S2). No other denitrated compound was detected. These particular dimers were not observed in the absence of NAD(P)H, i.e., when the release of nitrite was not significant. It is worth noting that in the study of Pak et al. (15), those authors identified an NADPH-dependent XenB reductase of *P. fluorescens* I-C associated with the production of these isomers. The NEM reductase of *E. coli* is similar to XenB (1) and has similar TNT catalytic activities in the presence of NAD(P)H (21). The involvement of NEM reductase was not demonstrated in our study. However, our experiments suggest such an involvement, because NAD(P)H was required and because we detected the production of isomers of amino-dimethyl-tetranitrobiphenyl. We have proposed a hypothetical biodegradation pathway of TNT by *E. coli* (see Fig. S3 in the supplemental material).

In summary, we have reported (i) the use of TNT as sole nitrogen source by *E. coli* and (ii) unequivocal evidence of TNT denitration by *E. coli*. We have also confirmed the formation of particular denitrated compounds. Further studies will determine which protein(s) mediates the TNT denitration reaction.

B.S. gratefully acknowledges the Fund for the Promotion of Research in Industry and Agriculture, Belgium, for providing a doctoral fellowship. Additional material support came from grant GLK-3-CT-2001-00345 (MADOX) funded by the EU under the 5th Framework Program.

We thank Marc Boutry (Unit of Physiological Biochemistry, Catholic University of Louvain) for the use of sonicator equipment. Finally, for his contribution to the experiments, we acknowledge Pierre-Rémy Schyns, a graduate student from the Paul Lambin Institute of Brussels, Belgium.

REFERENCES

1. **Blehert, D. S., B. G. Fox, and G. H. Chambliss.** 1999. Cloning and sequence analysis of two *Pseudomonas* flavoprotein xenobiotic reductases. J. Bacteriol. **181:**6254–6263.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Caballero, A., A. Esteve-Núñez, G. J. Zylstra, and J. L. Ramos.** 2005. Assimilation of nitrogen from nitrite and trinitrotoluene in *Pseudomonas putida* JLR11. J. Bacteriol. **187:**396–399.
4. **Caballero, A., J. J. Lázaro, J. L. Ramos, and A. Esteve-Núñez.** 2005. PnrA, a new nitroreductase-family enzyme in the TNT-degrading strain *Pseudomonas putida* JLR11. Environ. Microbiol. **7:**1211–1219.
5. **Caballero, A., and J. L. Ramos.** 2006. A double mutant of *Pseudomonas putida* JLR11 deficient in the synthesis of the nitroreductase PnrA and assimilatory nitrite reductase NasB is impaired for growth on 2,4,6-trinitrotoluene (TNT). Environ. Microbiol. **8:**1306–1310.
6. **Ederer, M. M., T. A. Lewis, and R. L. Crawford.** 1997. 2,4,6-Trinitrotoluene (TNT) transformation by clostridia isolated from a munition-fed bioreactor: comparison with non-adapted bacteria. J. Ind. Microbiol. Biotechnol. **18:**82–88.
7. **Eyers, L., B. Stenuit, S. El Fantroussi, and S. N. Agathos.** 2004. Microbial characterization of TNT-contaminated soils and anaerobic TNT degradation: high and unusual denitration activity, p. 51–54. *In* W. Verstraete (ed.), Proceedings of the European Symposium on Environmental Biotechnology, ESEB 2004. Taylor and Francis Group plc, London, United Kingdom.
8. **Eyers, L., B. Stenuit, R. Rozenberg, J.-L. Habib-Jiwan, and S. N. Agathos.** 2006. Denitration of 2,4,6-trinitrotoluene by a periplasmic extract from *Pseudomonas aeruginosa* ESA-5, abstr. 65, p. 136. International, European and Japanese Symposium of Environmental Biotechnology (ISEB/ESEB/JSEB 2006). Leipzig, Germany, 9–13 July 2006.
9. **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.
10. **Haïdour, A., and J. L. 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.
11. **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.
12. **Ingledew, W. J., and R. K. Poole.** 1984. The respiratory chains of *Escherichia coli*. Microbiol. Rev. **48:**222–271.
13. **Kurinenko, B. M., N. A. Denivarova, and G. Y. Yakovleva.** 2005. Sensitivity of various *Escherichia coli* strains to 2,4,6-trinitrotoluene. Appl. Biochem. Microbiol. (Russia) **41:**47–50.
14. **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.
15. **Pak, J. W., K. L. Knoke, D. R. Noguera, B. G. Fox, and G. H. Chambliss.** 2000. Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C. Appl. Environ. Microbiol. **66:**4742–4750.
16. **Ramos, J. L., M. M. González-Pérez, A. Caballero, and P. van Dillewijn.** 2005. Bioremediation of polynitrated aromatic compounds: plants and microbes put up a fight. Curr. Opin. Biotechnol. **16:**275–281.
17. **Reitzer, L.** 2003. Nitrogen assimilation and global regulation in *Escherichia coli*. Annu. Rev. Microbiol. **57:**155–176.
18. **Rohdich, F., J. Wungsintaweekul, M. Fellermeier, S. Sagner, S. Herz, K. Kis, W. Eisenreich, A. Bacher, and M. H. Zenk.** 1999. Cytidine 5′-triphosphate-dependent biosynthesis of isoprenoids: YgbP protein of *Escherichia coli* catalyzes the formation of 4-diphosphocytidyl-2-*C*-methylerythritol. Proc. Natl. Acad. Sci. USA **96:**11758–11763.
19. **Stenuit, B., L. Eyers, S. El Fantroussi, and S. N. Agathos.** 2005. Promising strategies for the mineralisation of 2,4,6-trinitrotoluene. Rev. Environ. Sci. Biotechnol. **4:**39–60.
20. **Strategic Environmental Research and Development Program.** 1999. Enzymes for degradation of energetic materials. Compliance CP-1078. U.S. Department of Energy, Richland, Wash. [Online.] http://www.serdp.org/research/CP/CP-1078.pdf.
21. **Williams, R. E., D. A. Rathbone, N. S. Scrutton, and N. C. Bruce.** 2004. Biotransformation of explosives by the old yellow enzyme family of flavoproteins. Appl. Environ. Microbiol. **70:**3566–3574.
22. **Yin, H., T. K. Wood, and B. F. Smets.** 2005. Reductive transformation of TNT by *Escherichia coli*: pathway description. Appl. Microbiol. Biotechnol. **67:**397–404.
23. **Yin, H., T. K. Wood, and B. F. Smets.** 2005. Reductive transformation of TNT by *Escherichia coli* resting cells: kinetic analysis. Appl. Microbiol. Biotechnol. **69:**326–334.
24. **Zabeau, M., and K. K. Stanley.** 1982. Enhanced expression of cro-β-galactosidase fusion proteins under the control of the P_R promoter of bacteriophage λ. EMBO J. **1:**1217–1224.