The Journal of Microbiology, April 2009, p. 220-224
DOI 10.1007/s12275-008-0108-0

Vol. 47, No. 2

## NOTE

# Comparative Analysis of 2,4,6-Trinitrotoluene (TNT)-Induced Cellular Responses and Proteomes in *Pseudomonas* sp. HK-6 in Two Types of Media

**Yun-Seok Cho[1], Bheong-Uk Lee[2], Hyung-Yeel Kahng[3], and Kye-Heon Oh[1]***

[1]*Department of Biotechnology, Soonchunhyang University, Asan 336-600, Republic of Korea*
[2]*Division of Biological Sciences, Kosin University, Busan 606-701, Republic of Korea*
[3]*Department of Environmental Education, Sunchon National University, Suncheon 540-742, Republic of Korea*

(Received April 28, 2008 / Accepted December 8, 2008)

**TNT-induced cellular responses and proteomes in *Pseudomonas* sp. HK-6 were comparatively analyzed in two different media: basal salts (BS) and Luria broth (LB). HK-6 cells could not degrade more than 0.5 mM TNT with BS medium, while in LB medium, they exhibited the enhanced capability to degrade as much as 3.0 mM TNT. Analysis of total cellular fatty acids in HK-6 cells suggested that the relative abundance of several saturated or unsaturated fatty acids is altered under TNT-mediated stress conditions. Scanning electron microscopy showed the presence of perforations, irregular rod formations, and wrinkled extracellular surfaces in cells under TNT stress. Proteomic analysis of soluble protein fractions from HK-6 cultures grown with TNT as a substrate revealed 11 protein spots induced by TNT. Among these, seven proteins (including Alg8, AlgB, NirB, and the AhpC/Tsa family) were detected only in LB medium containing TNT. The proteins AspS, Tsf, and assimilatory nitrate reductase were increasingly expressed only in BS medium containing TNT. The protein dGTPase was found to be induced and expressed when cells were grown in either type of TNT-containing media. These results provide a better understanding of the cytotoxicity and survival mechanism used by *Pseudomonas* sp. HK-6 when placed under TNT stress conditions.**

***Keywords*:** cellular responses, proteomics, alginate, *Pseudomonas* sp. HK-6, 2,4,6-trinitrotoluene

Microbial degradation of 2,4,6-trinitrotoluene (TNT) has been extensively studied (Fiorella and Spain, 1997; French *et al*., 1998; Oh and Kim, 1998; Pak *et al*., 2000; Lee *et al*., 2002), but information on TNT-induced stress shock proteins (SSPs) has been reported for only a few microorganisms (Chang *et al*., 2004; Ho *et al*., 2004). Proteome analysis is a powerful tool for investigating global changes in prokaryotic gene expression (Jungblut *et al*., 1999, 2000). Because 2-dimensional electrophoresis (2-DE) displays on a gel all bacterial soluble proteins expressed under specific culture conditions, high-throughput screening of induced proteins is possible. To date, proteome analysis has been performed on several bacteria including *Acinetobacter* sp. KS-1 (Kim *et al*., 2003), *Acinetobacter lwoffii* K24 (Kim *et al*., 2001, 2002), *Pseudomonas putida* KT2442 (Lupi *et al*., 1995), and *Stenotrophomonas* sp. OK-5 (Ho *et al*., 2004). Recently, enzymes and related proteins responsible for the degradation of aromatic compounds such as aniline, benzoate, 2,4-D, and TNT have been extensively studied by proteomic analysis. The results of such studies have provided environmental microbiologists with valuable information on the degradation of aromatic compounds (Park *et al*., 2001; Cho *et al*., 2002; Ho *et al*., 2004). In order to expand our knowledge of TNT-responsive SSPs capable of TNT degradation in *Pseudomonas* sp. HK-6 and increase our understanding of TNT-mediated toxicity and cellular responses, this study analyzed TNT-inducible stress shock proteomes in HK-6 cells.

*Pseudomonas* sp. HK-6 was cultivated in two different media: Luria broth (LB) containing TNT and basal salts (BS) medium containing TNT. The conditions of cultivation and maintenance of the HK-6 strain have been described previously (Chang *et al*., 2004). To monitor TNT degradation, residual TNT concentration was determined by reverse-phase HPLC as previously described (Ho *et al*., 2004). *Pseudomonas* sp. HK-6 can degrade TNT in BS medium and in LB medium under aerobic conditions (Fig. 1). With BS medium, 0.5 mM TNT was completely degraded within 144 h of incubation, and the culture pH changed from 7.2 to 7.1 (Fig. 1A). Surprisingly, in HK-6 cultures grown on LB medium containing 0.5 mM TNT, complete degradation was achieved within just 18 h (Fig. 1B). To further evaluate the capability of *Pseudomonas* sp. HK-6 in LB medium to degrade TNT, various concentrations of TNT were tested (Fig. 1C). Strain HK-6 grown on LB media was able to completely degrade TNT at all the concentrations tested, including the highest concentration of 3.0 mM TNT.

To examine fatty acids that shifted in response to TNT,

* To whom correspondence should be addressed.
(Tel) 82-41-530-1353; (Fax) 82-41-530-1350
(E-mail) kyeheon@sch.ac.kr

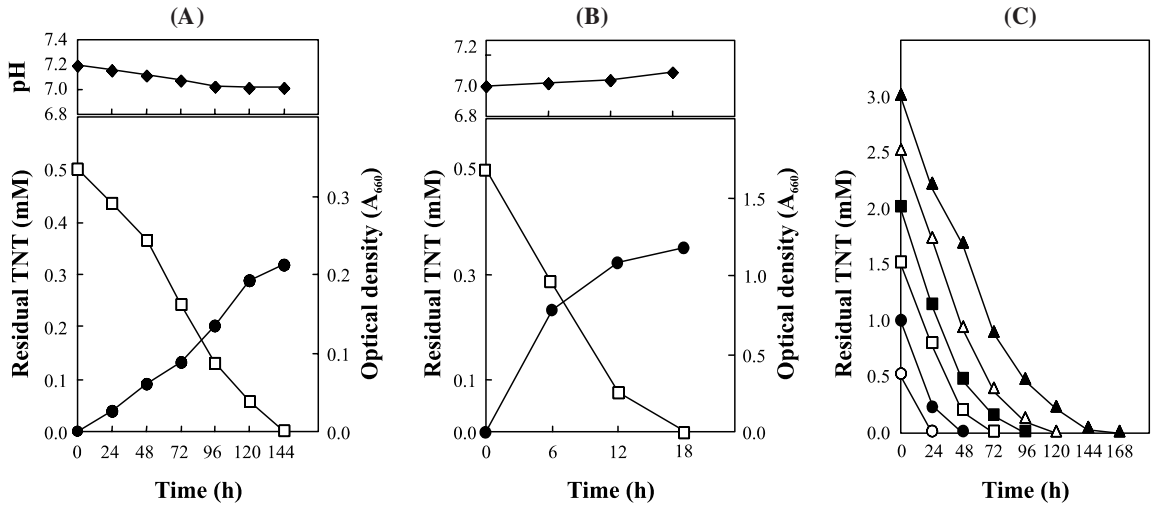


**Fig. 1.** Rate of TNT degradation by *Pseudomonas* sp. HK-6. HK-6 cells were grown with 0.5 mM TNT in BS medium (A) or with 0.5 mM TNT in LB (B), and then the cell density was measured at 660 nm (●). The rate of TNT degradation (□), and pH change (♦) over the incubation time course were determined in 250 ml flasks. Rates of TNT degradation in LB medium containing 0.5 mM (○), 1.0 mM (●), 1.5 mM (□), 2.0 mM (■), 2.5 mM (△), and 3.0 mM (▲) of TNT were similarly determined (C).

cells grown on tryptic soy broth (TSB) at 37°C for 24 h were collected, washed twice in potassium phosphate buffer (pH 7.0), and incubated in LB medium, BS medium containing 0.5 mM TNT, or LB medium containing 0.5 mM TNT. After 24 h, the fatty acid compositions were analyzed as described previously (Cho *et al*., 2002). As shown in Fig. 2, the dominant lipids in LB-grown HK-6 cells were 16:1 ω7c/15:0 iso 2OH, 16:0 and 18:1 ω7c/ω9t/ω12t, which decreased severely in cells exposed to TNT. Fatty acids 10:0 iso, 14:1 ω5c/ω5t and 17:0 cyclo were 10%, 8%, and 3% of the total cellular fatty acids in LB-grown cells, respectively, while in BS medium containing TNT, they accounted for 22%, 21%, and 8% of total cellular fatty acids, respectively. Some fatty acids exhibited similar shift patterns in HK-6 cells regardless of the medium used. The relative abundance of fatty acids 16:0, 16:1 ω7c/15:0 iso 2OH, and 18:1 ω7c/ω9t/

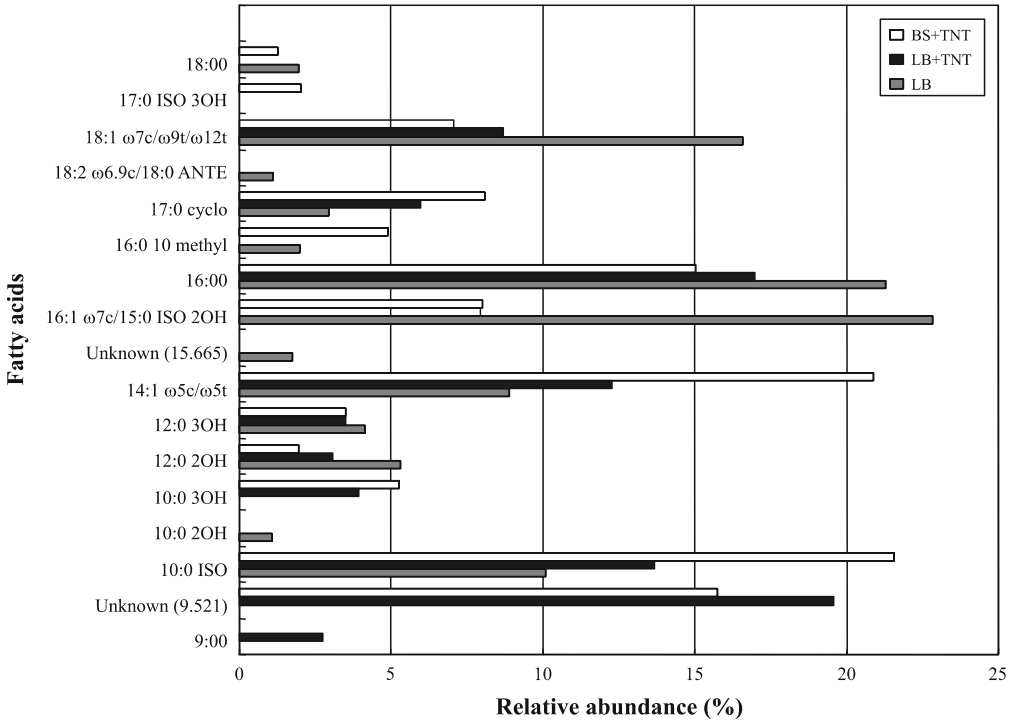


**Fig. 2.** Fatty acid profiles of *Pseudomonas* sp. HK-6 cells grown on LB, BS medium with TNT, or LB medium with TNT. Fatty acids were identified based on the retention of authentic references.

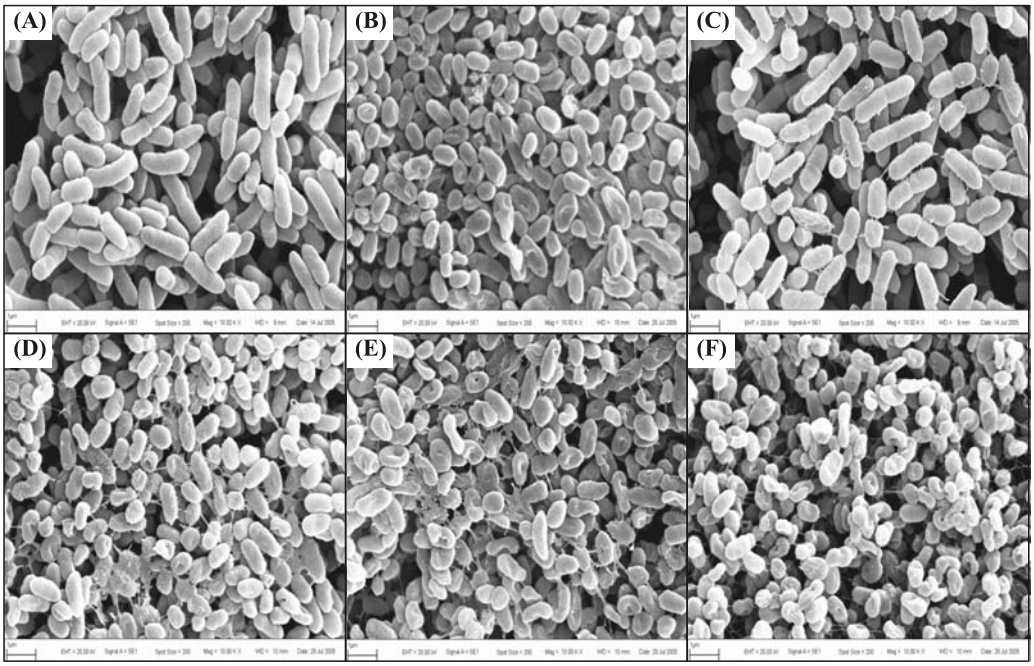


**Fig. 3.** Scanning electron micrographs of *Pseudomonas* sp. HK-6 cells grown on LB medium (A), 0.5 mM TNT plus BS medium (B), and 0.5 mM TNT plus LB medium (C). Lower panels show morphological characteristics of *Pseudomonas* sp. HK-6 cells treated with different concentrations of TNT on LB media: 1.0 mM TNT for 48 h (D), 2.0 mM TNT for 96 h (E), and 3.0 mM TNT for 144 h (F).

ω12t decreased in BS or LB media supplemented with TNT compared to unsupplemented LB medium, while the relative abundance of 10:0 iso increased when the media contained TNT.

In order to observe morphological cellular changes in LB medium with TNT, cells were exposed to 1∼3 mM TNT in LB for 48∼144 h. Cells were then fixed, dehydrated, and coated with gold and examined under a Hitachi S-2500C scanning electron microscope (Hitachi, Japan) as previously described (Ng *et al*., 1985). Cells grown in LB medium exhibited a typical rod shape with a smooth surface (Fig. 3A). Cells treated with 0.5 mM TNT in BS medium exhibited structural disruption in the cell envelope, as well as a preponderance of irregular rod formations with wrinkled surfaces (Fig. 3B). Cells grown on LB medium with 0.5 mM TNT showed some damaged rod formations, but most cells

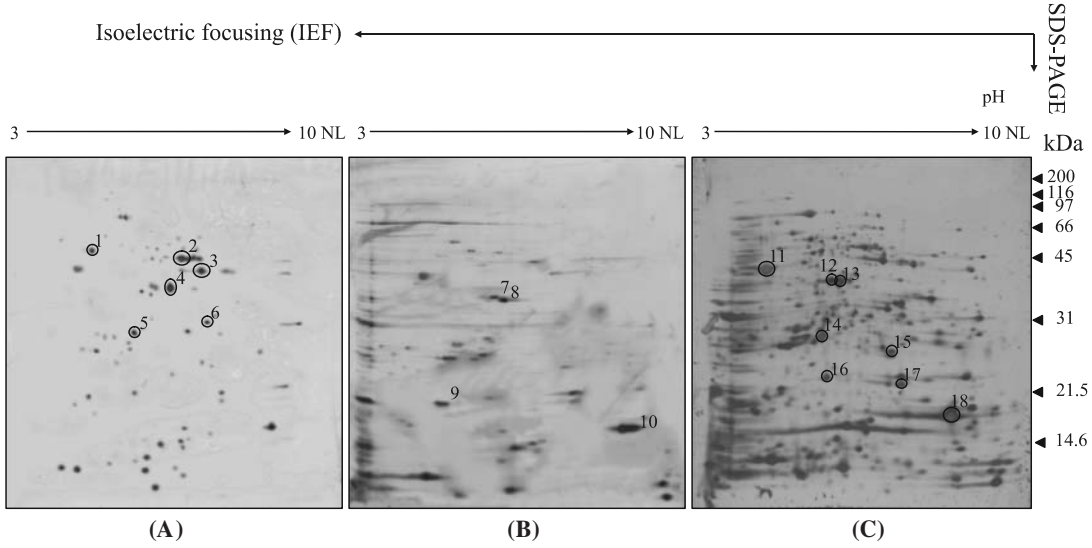


**Fig. 4.** 2-DE analysis of stress-induced proteins of *Pseudomonas* sp. HK-6 grown on LB medium (A), BS medium with 0.5 mM TNT for 96 h (B), and LB medium with 0.5 mM TNT for 12 h (C). For analytical 2-DE, 150 μg proteins were precipitated with ice cold acetone, resolubilized, and loaded on 18 cm long Immobiline DryStrip pH 3∼10 NL. NL, Non Linear.

appeared normal (Fig. 3C). Extracellular materials on HK-6 cells were observed in LB medium with TNT (0.5∼3.0 mM). Micrographs of HK-6 cells grown on LB medium with higher concentrations of TNT showed increased production of alginate on the surface (Fig. 3D∼F).

To determine the effect of TNT on the proteome of HK-6 cells, 2-DE and in-gel digestion were performed according to previously described methods (Heukeshoven and Dernick, 1985; Kim *et al.,* 2003; Ho *et al*., 2004). Molecular masses of the peptides were determined with a Voyager-DETM PRO Biospectrometry Workstation (Applied Biosystems, USA). Proteins were identified by peptide mass fingerprinting with the search program MASCOT (http://www.matrixscience.com) (Pertins *et al*., 1999). TNT-induced proteins were analyzed with HK-6 cells grown on LB, BS plus TNT, or LB plus TNT media (Fig. 4A∼C). Figure 4B shows significant induction of four proteins in response to TNT treatment in BS medium for 96 h. Figure 4C shows proteins induced for 12 h in LB containing 0.5 mM TNT. Assimilatory nitrate reductase, translation elongation factor Tsf, and aspartyl-tRNA synthetase were increasingly expressed only under TNT-induced stress conditions in BS medium (Table 1, Fig. 4B). Six proteins including Alg8 and AlgB, which are involved in alginate biosynthesis, were increasingly expressed only in LB plus TNT medium (Table 1, Fig. 4C). The only protein identified in both LB and BS media containing TNT was deoxyguanosine triphosphate triphosphohydrolase. Interestingly, expression levels of six proteins decreased in media containing TNT (Table 1, Fig. 4A).

*Pseudomonas* sp. HK-6 cells could not degrade more than 0.5 mM TNT with BS medium, whereas in LB medium, they exhibited the enhanced capability to degrade 3.0 mM TNT. As TNT concentrations increased in LB medium, complete TNT degradation was delayed. Nonetheless, LB medium clearly served as a good stimulant for TNT degradation by *Pseudomonas* sp. HK-6 cells. Our previous paper reported that more active bacterial growth and faster TNT degradation were observed in the presence of yeast extract (Oh and Kim, 1998). In fact, LB medium is composed of 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. The LB medium used in this study contains multiple carbon and nitrogen sources that can be utilized by the HK-6 strain. Therefore, it was speculated that the additional carbon and nitrogen sources in LB medium maintained continuous cell growth that, despite high concentrations of TNT, resulted in accelerated TNT degradation. In addition, assimilatory nitrate

**Table 1.** TNT-induced proteins identified by MALDI-TOF fingerprinting in *Pseudomonas* sp. HK-6

| Spot no. | Identified protein | Accession number | HK-6 growth conditions | | |
|---|---|---|---|---|---|
| | | | LB | BS+TNT | LB+TNT |
| Energy metabolism | | | | | |
| 2 | Aldehyde dehydrogenase, putative | AAN68197 | ↑[a] | - | - |
| 6 | Phosphogluconate dehydratase | AAN66635 | ↑ | - | - |
| 7 | Assimilatory nitrate reductase | AAN67324 | - | ↑ | - |
| 13 | Nitrite reductas, NirB | AAN67326 | - | - | ↑ |
| 15 | NADH dehydrogenase I, C, D subunit, NuoCD | AAN69704 | - | - | ↑ |
| Transport and binding proteins | | | | | |
| 1 | Outer membrane protein | AAN65899 | ↑ | - | - |
| 3 | Putrescine ABC transporter, periplasmic putrescine-binding protein, PotF-1 | AAN70745 | ↑ | - | - |
| 4 | General amino acid ABC transporter, periplasmic binding protein, AapJ | AAN66921 | ↑ | - | - |
| Protein synthesis ; tRNA amino acid acylation, translation factors | | | | | |
| 5 | Phenylalanyl-tRNA synthetase, alpha subunit, PheS | AAN68081 | ↑ | - | - |
| 8 | Translation elongation factor Ts, Tsf | AAN67213 | - | ↑ | - |
| 9 | Aspartyl-tRNA synthetase, AspS | AAN66837 | - | ↑ | - |
| Cell envelope | | | | | |
| 11 | Alginate biosynthesis protein Alg8 | AAN66911 | - | - | ↑ |
| 12 | Alginate biosynthesis transcriptional regulatory protein AlgB | AAN65767 | - | - | ↑ |
| 16 | UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate-D-alanyl-D-al anyl ligase, MurF | AAN66956 | - | - | ↑ |
| Cellular processes ; detoxification | | | | | |
| 14 | Antioxidant, AhpC/Tsa family | AAN66709 | - | - | ↑ |
| 17 | Outer membrane protein H1, OprH | AAN66809 | - | - | ↑ |
| Nucleotide and nucleoside interconversion | | | | | |
| 10,18 | Deoxyguanosine triphosphate triphosphohydrolase, dGTPase | AAN67716 | - | ↑ | ↑↑ |

[a] The arrows indicate up-regulation of protein expression level based on comparison of protein size

reductase and nitrite reductase NirB, which are involved in nitrogen metabolism, were highly expressed only in LB medium containing TNT. These enzymes play key roles in TNT metabolism (Caballero *et al*., 2005), and their higher cytoplasmic concentrations in LB medium might facilitate TNT degradation.

A number of studies have reported that aromatic hydrocarbons such as explosives and phenoxyherbicides have a toxic and ultimately fatal effect on cells due to the disruption of membrane components (Cho *et al*., 2000; Park *et al*., 2001; Cho *et al*., 2002; Chang *et al*., 2004; Ho *et al*., 2004). Interestingly, HK-6 cells grown on LB plus TNT were coated with a continuous layer of extracellular material called alginate (Lee *et al*., 2008). Cells grown on LB medium with TNT produced alginate, which was not produced by cells in unsupplemented LB medium (Fig. 3A). Alginate on cell surfaces was observed when cells were treated with higher concentrations of TNT such as 2.0 mM TNT for 96 h (Fig. 3E) and 3.0 mM TNT for 144 h (Fig. 3F).

The present study expands our fundamental knowledge of TNT stress response in *Pseudomonas* sp. HK-6. It is notable that alginate produced by HK-6 cells during cultivation increases the TNT tolerance of this bacterium. In our proteome analysis, alginate biosynthesis protein Alg8 and alginate biosynthesis transcriptional regulatory protein AlgB were highly expressed under TNT stress (Table 1). Indeed, a mutant lacking the *algA* gene (which encodes an enzyme that catalyzes the first step of alginate biosynthesis) was unable to produce extracellular fiber-like material (Lee *et al*., 2008). Alginate might be necessary for cell survival in harsh environments, such as TNT exposure.

This study also suggested that TNT induces different proteins depending on the culture media. A close connection was observed between cellular responses (e.g., shift in fatty acid composition in the cell membrane, production of alginate on the surface of the cell wall, and changes in expression of specific proteins in the cell) and cell survival of *Pseudomonas* sp. HK-6 grown in different media containing TNT. The TNT-induced proteins appear to play important roles in protecting cells and promoting cell survival.

This work was supported by grant (R01-2005-000-106080) from the Basic Research Program of the Korea Science & Engineering Foundation.

## References

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.

Chang, H.W., H.Y. Kahng, S.I. Kim, J.W. Chun, and K.H. Oh. 2004. Characterization of *Pseudomonas* sp. HK-6 cells responding to explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). *Appl. Microbiol. Biotechnol.* 65, 323-329.

Cho, Y.S., H.Y. Kahng, C.K. Kim, J.J. Kukor, and K.H. Oh. 2002. Physiological and cellular responses of the 2,4-D degrading bacterium, *Burkholderia cepacia* YK-2, to the phenoxyherbicides 2,4-D and 2,4,5-T. *Curr. Microbiol*. 45, 415-422.

Cho, Y.S., S.H. Park, C.K. Kim, and K.H. Oh. 2000. Induction of stress shock proteins DnaK and GroEL by phenoxyherbicide 2,4-D in *Burkholderia* sp. YK-2 isolated from rice field. *Curr. Microbiol*. 41, 33-38.

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

Heukeshoven, J. and R. Dernick. 1985. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* 6, 103-112.

Ho, E.M., H.W. Chang, S.I. Kim, H.Y. Kahng, and K.H. Oh. 2004. Analysis of TNT (2,4,6-trinitrotoluene)-inducible cellular responses and stress shock proteome in *Stenotrophomonas* sp. OK-5. *Curr. Microbiol*. 49, 346-352.

Jungblut, P.R., D. Bumann, G. Haas, U. Zimny-Arndt, P. Holland, S. Lamer, F. Siejak, A. Aebischer, and T.F. Meyer. 2000. Comparative proteome analysis of *Helicobacter pylori. Mol. Microbiol*. 36, 710-725.

Jungblut, P.R., U.E. Schaible, H.J. Mollenkopf, U. Zimny-Arndt, B. Raupach, J. Mattow, P. Halada, S. Lamer, K. Hagens, and S.H.E. Kaufmann. 1999. Comparative proteome analysis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG strains: Towards functional genomics of microbial pathogens. *Mol. Microbiol*. 33, 1103-1117.

Kim, S.I., S.J. Kim, S.H. Leem, K.H. Oh, S. Kim, and Y.M. Park. 2001. Site-directed mutagenesis of two cysteins (155, 202) in catechol 1,2-dioxygenase I1 of *Acinetobacter lwoffii* K24. *J. Biochem. Mol. Biol*. 34, 172-175.

Kim, S.I., S.J. Kim, M.H. Nam, S. Kim, K.S. Ha, K.H. Oh, J.S. Yoo, and Y.M. Park. 2002. Proteome analysis of aniline-induced proteins in *Acinetobacter lwoffii* K24. *Curr. Microbiol*. 44, 61-66.

Kim, S.I., S.Y. Song, K.W. Kim, E.M. Ho, and K.H. Oh. 2003. Proteomic analysis of the benzoate degradation pathway in *Acinetobacter* sp. KS-1. *Res. Microbiol*. 154, 697-703.

Lee, M.S., H.W. Chang, H.Y. Kahng, J.S. So, and K.H. Oh. 2002. Biological removal of explosive 2,4,6-trinitrotoluene by *Stenotrophomonas* sp. OK-5 in bench-scale bioreactors. *Biotechnol. Bioprocess Eng.* 7, 105-111.

Lee, B.U., S.C. Park, Y.S. Cho, and K.H. Oh. 2008. Exopolymer biosynthesis and proteomic changes of *Pseudomonas* sp. HK-6 under stress of TNT (2,4,6-trinitrotoluene). *Curr. Microbiol.* 57, 477-483.

Lupi, C.G., T. Colangelo, and C.A. Mason. 1995. Two-dimensional electrophoresis analysis of the response of *Pseudomonas putida* KT-2442 to 2-chlorophenol. *Appl. Environ. Microbiol*. 61, 2863-2872.

Ng, L.K., R. Sherburne, D.E. Taylor, and M.E. Stiles. 1985. Morphological forms and viability of *Campylobacter* species studied by electron microscopy. *J. Bacteriol*. 164, 338-343.

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.

Pak, J.W., K.L. Koke, 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.

Park, S.H., K.H. Oh, and C.K. Kim. 2001. Adaptative and cross-protective responses of *Pseudomonas* sp. DJ-12 to several aromatics and other stress shocks. *Curr. Microbiol*. 43, 176-181.

Pertins, D.N., D.J. Pappin, D.M. Creasy, and J.S. Cottrell. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20, 3551-3567.