# CONSTRUCTION OF 2,4,6-TRINITROTOLUENE- AND NITROBENZENE-CATABOLIZING BACILLI BY TRANSFORMATION AND GENE FUSION WITH PLASMID

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## INTRODUCTION

Nitroaromatic compounds are used in large quantity for production of plastics, explosives, pharmaceuticals, and pesticides (Bryant and DeLuca, 1991; Hallas and Alexander, 1983; Kedderis et al., 1988; Nishino and Spain, 1993). As a result, large amount of nitrobenzene is contaminating the environment at a rate of tens of millions of pounds annually (U.S. Environmental Protection Agency, 1978). In addition, nitrated polycyclic aromatic hydrocarbons are also formed during a variety of combustion processes causing serious environmental pollution (Rafii, et al., 1991). Reduction of the nitrogroup is a common first step in the biotransformation of nitroaromatic compounds, either leading to mineralization of the compounds (Groenwegen et al., 1992; Nishino and Spain, 1933) or leading to accumulation of dead-end products, many of them are cytotoxic or mutagenic (Bryant C. and McFlory. 1991; McCoy et al., 1990; Tatsumi et al., 1982; Narai, N., S. Kitamura, and K. Tasumi. 1984). Aerobic degradation of aromatic hydrocarbons by a microorganism was first demonstrated in the early 1900s.. Since then, various strains of bacteria and fungi capable of catabolizing aromatic hydrocarbons were isolated (Gibson et al. 1984). Biodegradation of aliphatic hydrocarbons (gasoline and diesel oil ) by bacteria were also developed contributing greatly in bioremediation of enviroment disaster by oil spill (Reisfeld et al. 1972; Rosenberg et al. 1979 a, b; Britton 1984; Shabtai et al. 1985; Yang et al. 1996a, b ). Study on the biotransformation of nitrated aromatics such as 2,4,6-trinitrotoluene (TNT) and nitrobenzene by bacteria demonstrated either aerobic or anaerobic metabolic pathway for degradation (Crawford, 1995; Marvin-Sikkema, 1994; Michaels and Gottschalk, 1995; Preuss et al. 1993; Spain, 1995; Stahl and Aust, 1995, Pasti-Grigsby et al., 1996). Reduction and release of nitro groups as nitrite was reported in aerobic microbial degradation of some nitroaromatics (McCormick et al. 1976). Our earlier report indicated that aliphatic hydrocarbon catabolism (Yang et al. 1996a, b), and aromatic hydrocarbon catabolism in bacilli bacteria (Yang et al. 1997) can be enhanced by transformation of bacilli with either plasmid $pTV_1Ts$ or $pLTV_1$ . The object of the current study is to investigate further whether the specific benzene-catabolizing capability of plasmids $pTV_1Ts$ or $pLTV_1$ (Yang et al. 1997) can enhance biodegradation of nitroaromatics in the transformed bacilli or not. Can the nitroaromatics-biodegradation capability of the transformed bacilli be enhanced by induction of denitrification catabolism with the inducer, sodium nitrate?

## MATERIALS AND METHODS

### Bacterial strains and plasmids

A bacterial strain, PY313, containing plasmid, $pTV_1Ts$ (Tn917Erm$^r$ Cm$^r$ Ts$^{rep}$ ) (Youngman et al. 1983, 1989) and a bacterial strain PY1177, containing plasmid, $pLTV_1$ (Tn917Erm$^r$Cm$^r$Amp$^r$Tc$^r$ LacZ$^+$ Ts$^{rep}$)(Camilli et al. 1990) were obtained from Dr. Youngman. The host cells for the both plasmids were originated from BD170 (trpC2 thr5), one of B. subtilis 168 derivatives (Dubnau et al. 1969). The recipients of the plasmid in the current transformation experiment were B. subtilis 168 prototrophic from the Bacillus Genetic Stock Center (BGSC) and a strain of wild type selenite-resistant bacillus (MR1) which was isolated from Mississippi River. The selenite-resistant, MR-1 was classified into the genus of Bacillus mycoides (Yang et al. 1994) according to the difference in carbon source utilization by microplate incubation method of Biolog Inc. (Miller et al. 1991; Klinger et al. 1992).

**Culture media, selection media and reagents**

Luria-Bertani broth (LB) containing 0.5% NaC1, 0.8% tryptone, 0.3% yeast extract was used for regular liquid culture. Tryptose Blood Agar Base (TBAB) medium containing 0.5% NaC1, 1.0% tryptose, 0.3% beef extract and 1.5% agar was used for regular agar plate culture. Selection media for bacteria resistant to antibiotics erythromycin and chloramphenicol were made by inclusion of 2 $\mu$g/ml erythromycin and 12.5 $\mu$g/ ml chloramphenicol in LB (as LBEC) or in TBAB (as TBABEC). Selection media for bacteria resistant to antibiotics erythromycin and tetracyclin were made by inclusion of 2 $\mu$g/ml erythromycin and 12.5 $\mu$g/ml tetracyclin in LB (as LBET) or in TBAB (as TBABET). Most frequently used basic medium 2$\beta$ agar (BM2$\beta$ agar) for this study was made by mixing of sterilized chemical solutions into intensively washed and autoclaved agar at about 70°C before making agar plate. The final concentrations of chemicals in BM2$\beta$ agar are: 79.8 mM for $K_2HPO_4$, 44.1 mM for $KH_2PO$, 32 mM for $NaNO_3$, 6.8 mM for sodium citrate, 0.8 mM for $MgSO_4$, 0.1 mM for $Ca(NO_3)_2$, 0.1 mM for $MnCl_2$, 0.001 mM in $FeSO_4$, 0.32 mM for $Na_2MoO_4$, and 2% for agar. BM3$\beta$ agar was prepared from all chemicals listed for preparation of BM2$\beta$ agar except $NaNO_3$ and $Na_2MoO_4$ . For preparation of regular basic medium, BM1$\alpha$ agar, in addition to all chemicals and agar in BM3$\beta$ agar, 151.4 mM of $NH_4SO_4$ and 2% dextrose were included. In BM1$\alpha$ dextrose was used as the only carbon source for metabolism. In special basic media, BM2$\beta$ or BM3$\beta$, either aliphatic or aromatic hydrocarbons or their derivatives will be added to use as the carbon source depending on the nature of experiment. For testing of 2,4,6-trinitrotoluene(TNT)-catabolizing capability, 50% TNT in dimethylsulfoxide (DMSO) was applied 1 to 8 spots (50$\mu$m per spot) on BM2$\beta$ or on BM3$\beta$ agar plate. For testing of nitrobenzene(NB)-catabolizing capability of bacilli either BM2$\beta$ agar or BM2$\beta$ agar was mixed with 1% NB before pouring into petri dish for formation of agar plate. Most of the chemicals were supplied either from Sigma or from Fisher Co.. TNT was purchased from the Chemical Service Co.

**Preparation of plasmids**

For isolation of $TV_1Ts$, a single colony of PY313 was cultured in LBEC containing erythromycin (2 $\mu$g/ml) and chloramphenicol (12.5 $\mu$g/nil) at 30°C with 250 rpm for 24 hours. After centrifugation at 3000 rpm for 20 minutes, bacterial pellet was obtained for preparation of plasmid according to a modified method using alkaline lysis of host bacteria (Lee et al. 1990). The purified plasmid DNA was precipitated and kept in 70% ethanol until the time of further experiment. For isolation of plasmid, $pLTV_1$, a single colony of PY1177 was cultured in LBET containing erythromycin (2 ug/ml) and tetracyclin (12.5 ug/nil) at 30°C with 250 rpm for 24 hours. After centrifugation at 3000 rpm for 20 minutes, bacterial pellet was obtained for preparation of plasmid, $pLTV_1$ according to a modified method using alkaline lysis of host bacteria (Lee et al. 1990). The purified plasmid $pLTV_1$ was precipitated and kept in 70% ethanol until the time of further experiment.

**Transformation of B. subtilis prototrophic or wild tvpe selenite-hvnerresistant bacilli (MR1) with $pTV_1Ts$ or $pLTV_1$**

After overnight culture of a single colony of 168 prototrophic or MR1 in 12 ml LB at 30° C for 16 hours, a stationary phase of bacterial growth was obtained. After centrifugation at 3000 rpm for 20 minutes, a cell pellet was isolated. One ml of LB was used to resuspend the cell pellet, another ml was used to dissolve plasmid , $pTV_1$ Ts following removal of 70% ethanol from the plasmid storage tube. After mixing cell suspension with the plasmid, $pTV_1Ts$ suspension, the mixture was electroporated with 400 DCV for 100 msec. Shaking culture of the electroporated mixture was continued for another 6 to 12 hours in a smaller tube (13 x 100 mm) . Thereafter, 200 $\mu$l of the mixture was spread-cultured on BM1$\alpha$EC agar plate containing erythromycin (2 $\mu$g/ml) and chloramphenicol (12.5 $\mu$g/ml) for formation of colonies on the agar plate at 28° C (Yang et al. 1996). For preparation of transformed bacilli for test, a single colony was isolated into 42.5 ml of LBEC broth for shaking culture at 29°C for 24 hours. Following the addition of 7.5 ml glycerol, the cell preparation in LBEC containing 15% glycerol was divided into tubes, 5 ml per tube for storage at −80°C until next

experiment. For transformation of bacilli with plasmid $pLTV_1$, a colony of 168 prototrophic or MR1 was cultured in 12 ml LB broth at 29°C for 16 hours to stationary phase. After centrifugation at 3000 rpm for 20 minutes the supernatant was removed. One ml of the supernatant was used to resuspend the pellet, another ml of the supernatant was used to resuspend the plasmid, $pLTV_1$. After mixing cell suspension with the plasmid ($pLTV_1$) suspension, the mixture was electroporated with 400 DCV for 100 msec. Shaking culture of the treated mixture was continued for another 6 hours in a smaller tube (13 x 100 mm) . Thereafter, 200 μl of the mixture was spread-cultured on BM1αETagar plate (Table 1) containing erythromycin (2 μg/ml) and tetracyclin (12.5 μg/ml) for formation of colonies on the agar plate at 28° C (Yang et al. 1996). For preparation of transformed bacilli for test, a single colony was isolated into 42.5 ml of LBET broth for shaking culture at 29°C for 24 hours. Following the the addition of 7.5 ml glycerol, the cell preparation in LBET containing 15% glycerol was divided into tubes, 5 ml per tube for storage at −80°C until next experiment. A derivative of B. subtilis 168 prototrophic transformed by $pTV_1$ Ts was designated as WH-0, and another strain of wild type bacillus MR-1 (B. mycoides) transformed by the same plasmid, $pTV_1$ Ts was designated as WH-1 respectively. Similarly, a strain of B. subtilis 168 prototrophic transformed by $pLTV_1$ was designated as WH-2, and another strain of wild type bacillus MR-1 (B. mycoides) transformed by the same plasmid, $pTV_1$ Ts was designated as WH-3 respectively.

**Assessment of TNT-catabolism by test bacilli on BM2β or BM3β agar plate at permissive temperature or non-permissive temperature for plasmid activity**

All test bacilli were cultured for 24 hours in shaker incubator at 200 rpm and 28°C to late stationary phase in LBEC or LBET containing antibiotics. Following the addition of 15% glycerol, the test bacilli were frozen in a deep freezer at −80°C until the time of use. For the test, the frozen stock of test bacilli was thawed and resuspended homogenously to apply 8 LB spots ( 50 μm per spot) on the agar plate. Following incubation of the agar plate for 24 hours at permissive temperature (28°C) or non-permissive temperature (38°C), 50 % TNT in dimethylsulfoxide (DMSO) was applied to 8 spots (50 μm per spot) between LB spots on the plate. Following the application of LB spots and TNT spots on BM2β or BM3β agar plate, plates were incubated for another 14 days to examine color formation and colony formation on the plates .

**Assessment of NB-catabolism by test bacteria**

For assessment of NB-catabolism by mutant bacilli on the BM2βNB, or BM3βNB agar plates , the frozen stocks of prepared bacilli in LB medium containing 15% glycerol, was applied 50 μl per spot in 4 to 5 evenly-distributed locations on the agar plate containing 1% NB. Following 14 days of incubation at the temperature of 28°C, the territory of bacterial growth was examine by color photograph with or without the help of either Giemsa or Wright staining.

## RESULTS

**Color formation and Colony formation for assessment of TNT-catabolism**

Current method used in the assessment of TNT-catabolism is simple and effective because significant color changes in the LB spot can be easily recognized for assessment of the TNT-degradation . In addition, the colony formation in the LB spot can be used for assessment of cell growth coupled TNT-catabolism and successful detoxification of medium environment. Starting from longer wavelength near to 700nm (red) for the TNT to shorter wavelength for dinitrotoluene (DNT), nitrotoluene (NT), and others intermediates of metabolic products, colorful changes could be observed and expected from TNT-biodegradation. At the background of color change is the chemically synthesized basic media which was almost colorless and semitransparent, providing best environment for the color change to be observed.

**TNT-catabolism at permissible temperature 28°C**

When native bacilli of either B. subtilis 168 prototrophic or MR-1 (B. mycoides) were used for 14 days of incubation at 28°C on BM2β agar plate with the addition of TNT spots a day later, neither color formation nor colony formation was observed on the LB spots in the control experiment (Groups 1

and 6, Tables 1 and 2). Nevertheless, when pTV$_1$Ts-transformed bacilli (Groups 2 and 7, Table 1) or pLTV$_1$-transformed bacilli (Groups 2 and 7, Table 2) were applied to the LB spots in the BM2β plate with the addition of TNT spots , the response of bacteria to the TNT diffused from the adjacent TNT-spots wer very significant. A gradually increased intensity of brown color started to appear on the LB spots following 2 to 3 days of incubation. The brown-colored colonies appeared in 1 to 2 weeks of incubation at permissive temperature for plasmid replication (28°C) (Groups 2 and 7, Tables 1 and 2). In contrast, when the transformed bacilli were cultured in the BM3βTNT agar plate in the absence of sodium nitrate for induction of denitrification catabolism, the formation of brown color was seen on the LB spots without any visible colony formation (Groups 3 and 8, Tables 1 and 2).

**TNT-catabolism operated by gene-fusion of plasmid with chromosome at non-permissible temperature 38°C**

When the transformed bacilli in LBEC (WH-0 or WH-1) or in LBET(WH-2 or WH-3) medium were applied to LB spots on BM3β agar plate (in the absence of sodium nitrate) for incubation at non permissive temperature at 38°C for a day, mutation were induced by gene fusion between plasmid and chromosome. Following such treatment, some mutants became capable of catabolizing TNT with colony formation in the absence of sodium nitrate for denitrification. On such occasion, some mutants were able to catabolize TNT in BM2β at non-permissible temperature also (Groups 4 and 9 in Tables 1 and 2). Some mutants were able to grow into colonies in BM3βTNT using TNT as the only nitrogen source (Groups 5 and 10, Tables 1 and 2).

**NB-catabolism operated by mutant bacilli isolated from BM2βTNT or BM3βTNT agar plates.**

Transformed bacilli were also incubated for 24 hours at non-permissive temperature (38°C) for mutagenic treatment on either BM2β agar plate or BM3β agar plate followed by addition TNT spots on plates (Groups 4, 5, 9, 10, Tables 1 and 2) for isolation of mutants colonies. Some of isolated mutant colonies had increased growing capability out of LB spots in either BM2βTNT agar plate or BM3βNB agar plate. On such occasions, mutant bacilli were capable of growing on BM3βNB agar plate independently without the application of LB spots . Four of such mutants were isolated from 52 of mutants appeared on Group 10 Table 2.

## DISCUSSION

It is extremely difficult for bacteria to grow in the toxic environment TNT or other nitroaromatics in the medium. Enzymatic modification of nitro group in the aromatic compounds to less toxic chemicals appeared to be the necessary first step for temporary survival of bacteria. If degradation of such nitro groups can be coupled with energy-productive phosphorylation by electron transfer chain in denitrification (nitrate_ nitrite_ nitric_ oxide_nitrous oxide_nitrogen), it will be favorable for bacteria not only for bacterial survival but also for growth. As it can be seen from neither color nor colony formation of both 168 prototrophic (B. subtilis ) and MR-1 (B. mycoides) on the BM2βTNT agar plate in a incubator controlled at permissive temperature (28°C) (Groups 1 and 6, Tables 1 and 2). The bacterial enzymes of the native bacilli, which had been induced and activated by the inducer, sodium nitrate in BM2βTNT agar plate, appeared to have no capability for biodegradation of TNT. In the absence of the inducer, sodium nitrate in BM3β agar plate, to activate denitrification enzymes in the transformed bacilli , the transformed bacilli can perform incomplete biodegradation of TNT without colony formation . There are no coupling of metabolic reaction between TNT-biodegradation and cell growth (Group 3, Tables 1 and 2) . When pTV$_1$Ts -transformed bacilli or pLTV$_1$ -transformed bacilli were incubated at the permissive temperature (28°C) in the presence of the inducer, sodium nitrate in BM2βTNT agar plate, catabolism of TNT-degradation was well coupled to cell growth and colony formation of transformed bacilli in LB spots (Groups 2 and 7 in Tables 1 and 2). Further treatment of transformed bacilli in the non-permissible temperature at 38°C resulted in mutation of bacilli for rearrangement of new enzyme systems capable of catabolizing TNT in the presence (Groups 4 and 9, Tables 1 and 2) or absence (Groups 5 and 10, Tables 1 and 2) of inducer, sodium nitrate.

Earlier study by Duque et al (1993)demonstrated that Pseudomonas sp. clone A can grow on medium using TNT as nitrogen source and fructose as carbon sourcce. By transformation of the Ps. clone A with TOL plasmid, pWWO-Km, the hybrid were capable of mineralizing TNT as the only carbon

source and nitrogen source. In the current study, enhancement of benzene- and gasoline-catabolizing capability of bacilli by transformation with either plasmid $pTV_1Ts$ or pLTV1, leaded to the conversion of transformed bacilli capable of catabolizing TNT in the BM2β agar plate with the help of inducer, sodium nitrate, to induce denitrification catabolism. Further mutagenic treatment of the transformed bacilli (WH-3) with non-permissible temperature at 38°C lead to the creation of mutants (Groups 9 and 10, Tables 1 and 2) . Few mutants, WH-3M#49 and WH-3M#50 were capable of growing extensively out of the LB spots on the BM2βTNT or BM2βNT agar plate. Those mutants are capable to mineralize TNT or NB as the double nitrogen source or as the single carbon source. Those hybrid bacterial strains were also capable of growing on BM3βTNT or BM3βNB using TNT or NT as the single nitrogen source and the single carbon source due to genetic change caused by mutation. Most nitroaromatics are recalcitrant to bacteria and reports of their use as carbon sources by microorganisms were rare (Fernando et al. 1990). When they were used, biodegradation was never complete either because part of the products remained unattacked or because it was converted to partially reduced forms. The biotreatment of waste streams containing polynitroaromatics and other pollutants of this type usually required concerted action by series of microorganisms to achieve full degradation (Nay et al., 1974;Osmod and Klausmeier, 1972;Taxler et al., 1974) . Alternatively, a single organism capable to eliminate nitroaromatics could be produced by appropriate recruitment of catabolic functions, as described for the metabolism of haloaromatics (Lehrbach et al., 1984; , Ramos et al. 1986; Reinecke and Knackmuss, 1979).

## CONCLUSION

Using color and colony formation in the LB spots for testing metabolic response of test bacilli to TNT diffused from the nearby TNT-spots, the TNT-catabolizing capability of transformed bacilli or hybrid can be evaluated. A brown color-formation together with numerous colony formations on the bacterial-medium spots suggested a significant coupling of TNT-catabolism with a bacterial growth on the medium. Transformation of bacilli with plasmids capable for catabolizing aromatic hydrocarbon in our experiment appeared to be the most important first step successful biodegradation of nitroaromatics . Induction of denitrification catabolism by the inducer, sodium nitrate for switching regular oxidative phorphorylation to nitrate-directed denitrification appeared to be the second important procedure for biodegradation of nitroaromatics. By combination of two necessary procedures in the current experiment, successful TNT- catabolism by the $pTV_1Ts$ -transformed bacilli ( WH-0 and WH-1) or by the $pLTV_1$ -transformed bacilli ( WH-2 and WH-3) were achieved at the permissive temperature for the plasmid activity (28°C). Further treatment of transformed bacilli at non-permissive high temperature at 38°C on BM3β, resulted in the production of mutant capable of denitrifying TNT or nitrobenzene without nitrate inducer. Some of mutants were capable of catabolizing TNT as the only source of nitrogen and carbon source in the medium.

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## REFERENCES

Bryant, C., and M. DeLuca. 1991. Purification and characterization of and oxygen-insensitive NAD(P)H nitroreductase from Enterobacter cloacae. J. Biol. Chem. 266: 4119-4125.

Bryant, C., and W. D. McElroy. 1991. Nitroreductases. P. 291-304. In F. Muller (ed.), Chemistry and Biochemistry of flavoenzymes. Vol. II. CRC Press. Boca Baton, Fla.

Crawford, R.L. 1995. Biodegradation of nitrated munition compounds and herbicides by obligatory bacteria. P. 87-98. In J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenu Press. New York.

Duque, E., A. Haidour, F. Godoy, and J. L. Ramos. 1993. Construction of Pseudomonas hybid strain that mineralize 2,4,6-trinitrotoluene J. Bacteriology 175: 2278-2283.

Fernando, T., J.A. Bumpus, and S.D. Aust. 1990. Biodegradation of TNT (2,4,4-trinitrotoluene) by Phanerochaete chrysosporium. Appl. Environ. Microbiol. 56:

1666-1671.

Groenewegen, P. E., J. P. Breeuwer, J. M. L. M. van Helvoot, A. A. M. Langenhoff, F. F. de Vries, and J. A. M. de Bont. 1992. Novel degradative pathway of 4-nitrobenzoate in Comamons acidovorans NBA-10. J. Gen, Microbiol. 138: 1599-1605.

Hallas, L. E., and M. Alexander. 1983. Microbial transformation of nitroaromatic compounds in sewage effluent. Appl. Environ. Microbiol. 45: 1234-1241.

Kedderis, G. L., L. S. Argenbright, and G. T. Miwa. 1988. Mechasnism of reductive activation of a 5-nitroimidazole in by flavoproteins: model studies with dithionite. Arch. Biochem. Biophys. 262: 40-48.

Lehrbach, P. R., J. Zeyer, W. Reineke, H.-J. Knackmuss, and K. N. Timmis. 1984. Enzyme recruitment in vitro: use of cloned genes to extend the range of haloaromatics degraded by Pseudomonas sp. strain B13. J. Bacteriol. 158: 1025-1032.

Lewis, T.A., Goszczynski, S., R. L. Crawford, R.A. Korus, and W. Admassu. 1996. Products of anaerobic 2,4,6-trinitrotoluene(TNT) transformation by Clostridium bifermentans. Appl. Environ. Microbiol. 62: 4669-4674.

Marvin-Sikkema, F.D., and J. A. M. de Bont. 1994. Degradation of nitroaromatic compounds by microorganisms. Appl. Microbiol. Biotechnol. 42:499-507.

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.

McCoy, E. C., H. S. Rosenkranz, and P. C. Howard. 1990. Salmonella typhimurium TA100Tn5-1012, a strain deficient in arythydroxylamine O-esterificase exhibit a reduced nitroreductase activity. Mutat. Res. 243: 141-144.

Michels, J., and G. Gottschalk 1995. Pathway of of 2,4,6,-trinitrotoluene(TNT) degradation by by Phanerochaete chrysosorium , p. 135-149. In J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenum Press. New York.

McGany, J. D., and D. W. Foster. 1980. Regulation of hepatic fatty acid oxidation and ketone body production. Ann. Rev. Biochem. 49:395

Mello-Filho, A. C., and R. Menegluni. 1984. In vitro formation of single-strand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss Reaction. Biochemica et Biophysica Acta. 781:56-63.

Miller, J. M. and D. L. Rhoden. 1991. Preliminaiy evaluation of Biolog, a carbon source utilization methods for bacterial identification. J. of Clinical Microbiology. 29:1143-1147.

Nay, K. W., C. W. Randall, Jr., and P. W. King. 1974. Biological treatability of trinitrotoluene manufacturing waste-water. J. Water Pollut. Control Fed. 46: 485-497.

Nishino, S.F., and J. C. Spain. 1993. Degradation of nitrobenzene by Pseudomonas pseudoalcaligenes. App. Environ. Microbiol. 59: 2520-2525.

Narai, N., S. Kitamura, and K. Tasumi. 1984. A comparative study on 1-nitropyrene and nitrofurazone reductases on Escherichia coli. J. Pharm. Dyn. 7: 407-413.

Osmod, J. L., and R. E. Klausmeier. 1972. The microbial degradation of explosives. Dev. Ind. Microbiol. 14: 247-252.

Pasti-Grigsby. M.B., T. A. Lewis, D. L. Crawfoprd, and R. L. Crawford. 1996. Transformation of 2,4,6-trinitrotoluene (TNT) by actinomycetes isolated from TNT-contaminated and uncontaminated environments. Appl. Environ. Microbiol. 62: 1120-1123.

Perkins, 1. B., and P. Youngman 1986. Construction and properties of Tn9 I 7-lac, a transposon derivative that mediates transcriptional gene fusion in Bacillus subtilis. Proc. Nail. Acad. Sc. USA 83:140 144

Preuss, A., and P. G. Rieger. 1995. Anaerobic transformation of trinitrotoluene and other nitroaromatic compounds. P. 69-85. In J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenum Press. New York.

Rafii, F., W. Franklin, R. H. Heflich and C. E. Cerniglia. 1991. Reduction of nitroaromatic compounds by anaerobic bacteria isolated from the human gastrointestinal tract. Appl. Environ. Microbiol. 57: 962-968.

Ramos, J. L., A. Stolz, W. Reineke, and K. N. Timmis. 1986. Altered effector specificities in regulators of gene expression: TOL plasmid xy/S mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. Proc. Natl. Acad. Sci. USA 83: 8467-8471.

Reinecke, W., and H. J. Knackmuss, 1979. Construction of haloaromatic-utilizing bacteria. Nature (London) 277: 385-386

Reisfeld, A., E. Rosenberg, and D. Gutnick. 1972. Microbial degradation of crude oil: Factors affecting the dispersion in sea water by mixed and pure cultures. App. Microbiol. 24:363-368.

Rosenberg, E., A. Zuckerberg, C. Rubinovit.z, and D.L. Gutnick. 1979a. Emulsifier of Arthrobacter RAG1:isolation and emulsifing properties. App. Environ. Microbiol. 37:402-408.

Rosenberg, E., A. Perzy, D. T. Gibson, and D. L. Gutnick. 1 979b. Emulsifier of Arthrobacter RAG-i: specificity of hydrocarbon substrate. Appl. Environ. Microbiol. 37:409-413.

Shabtai, Y. and D. L. Gutnick. 1985. Exocellular esters and emulsan release from the cell surface Acinetobacter calcoaceticus. 1. Bacteriol. 161:117~1181.

Spain, J. C. 1995. Bacterial degradation of nitroaromatic compounds under aerobic conditions. P. 19-35. In J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenum Press. New York.

Stahl, J.C. and S. Aust 1995. Biodegradation of 2,4,6-trinitrotoluene by the white rot fungus Phanerochaete chrysosporium, p. 117-133. In J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenum Press. New York.

Traxler, R. W., E. Wood, and J. M. Delaney. 1974. Bacterial degradation of α-TNT. Dev. Ind. Microbiol. 16: 71-76.

U. S. Environmental Protection Agency. 1978. Nitrobenzene Initial report of the TSCA interagency testing committee to the administrator. EPA 560-578/001. US. Environmental Protection Agency. Washington, D.C.

Yang, W. H., A. Baaree, J. R. Yang, and A. Yee. 1994. High selenium concentration and selenit hyperresistant bacteria in the lower stream of Mississippi River. In Proceedings of the 24th Mississippi Water Resources Conference 24:37-45

Yang, W. H., and J. R. Yang. 1996a. Transformation of Bacillus Subtilis into hydrocarbon-resistant bacillus In Proceedings of the 26th Mississippi WateResources Conference 26: 250-257.

Yang, W. H., and J. R. Yang. 1996b. Transformation of selenite-hyperresistant Bacillus subtilis into hydrocarbon-resistant bacteria for bioremediation a oil-contaminated site. In Proceedings of the 12th International Conference on Advanced Science and Technoloav, 221-236.

Youngman, P., J. V. Perkins, and R Losich. 1983. Genetic transposition and insertional mutagenesis in Bacillus subtilis with Streptococcus faetalis transposon Tn9 17. PNAS. 80: 2305.

Youngman, P., H. Poth, B. Green, K. York, G. Olmedo, and K. Smlth. 1989. Method for genetic manipulation, cloning, and functional analysis of Sporulation Gene in Bacillus subtilis, In "Regulation of Procarvotic Development, eds. Smith, I., R. A. Slepecky, and P. Setiow". Washington, D. C. Amer. Soc. for Microbiology. 65.

Britton, L. N. 1984. Microbial Degradation of Aliphatic Hydrocarbons, in *Microbial Degradation of Organic Compounds*, D. T. Gibson (ed.). Marcel Dekker, Inc., New York pp. 89—129.

Gibson, D. T., and V.. Subramanian. 1984. MicrobiaL Degradation of Aromatic Hydrocarbons, in *Microbial Degradation of Organic Compounds*, D. T. Gibson, (ed.). Marcel Dekker, Inc., New York, pp. 18-252.

Table 1. Growth characteristics of $pTV_1Ts$-transformed bacilli (WH-0 and WH-1) on BM2βTNT or BM3βTNT agar plate after application of bacteria (in 8 LB spots) on Day 1 and TNT (in 4~8 spots) on Day 2 for incubation culture at 28°C or 38°C for 14 days .

| Exp. No. | Test Bacilli* | Incub. Temp. | Medium | No. of plate | LB-spot color | Colony and color | No. of colonies ($\mu \pm \sigma_{n-1}$) per spot | per plate | Colony form. Rate **** (per million of bacteria) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 168-prot (control) | 28°C | BM2βTNT | 3 | No color | No colony | 0.0 | 0.0 | 0.0 |
| 2 | WH-0** | 28°C | BM2βTNT | 3 | Brown | Brown colonies | 6.7 ±4.4 | 74.5 ±6.7 | 2.02/ million |
| 3 | WH-0 | 28°C | BM3βTNT | 3 | Brown | No colony | 0.0 | 0.0 | 0.0 |
| 4 | WH-0 | 38°C | BM2βTNT | 3 | Brown | Brown colonies | 3.4 ±3.2 | 27.2 ±9.5 | 0.74/ million |
| 5 | WH-0 | 38°C | BM3βTNT | 3 | Brown | Brown colonies | 4.8 ±2.6 | 38.4 ±8.6 | 1.04 /million |
| 6 | MR-1(control) | 28°C | BM2βTNT | 3 | No color | No colony | 0.0 | 0.0 | 0.0 |
| 7 | WH-1*** | 28°C | BM2βTNT | 3 | Brown | Brown colonies | 7.1 ±4.8 | 56.8 ±5.6 | 1.86/ million |
| 8 | WH-1 | 28°C | BM3βTNT | 3 | Brown | No colony | 0.0 | 0.0 | 0.0 |
| 9 | WH-1 | 38°C | BM2βTNT | 3 | Brown | Brown colonies | 5.4 ±2.7 | 35.0 ±14.5 | 1.15 /million |
| 10 | WH-1 | 38°C | BM3βTNT | 3 | Brown | Brown colonies | 4.8 ±2.9 | 38.4 ±12.8 | 1.26 /million |

* Test bacteria in LBEC medium containing at the late stage of stationary phase was frozen in the deep freezer after the addition of 15% glycerol. The frozen stocks were removed from the deep freezer and thawed just before the experiment.

** B.subtilis 168 prototrophic transformed with plasmid $pTV_1Ts$ was designated as WH-0

*** MR-1 (B. mycoides ) transformed with plasmid $pTV_1Ts$ was designated as WH-1.

**** Colony formation rate per million is calculated from the number of colony formation on test medium per that on the TBAB plate with the dilution factor of a million. It is based from the dilution experiment that 1 million dilution of test bacteria WH-0 will form 92 colonies/ml on TBAB plate and that the test bacteria of WH-1 in 1 million times dilution will form 76 colonies per ml on the TBAB plate.

Table 2. Growth characteristics of $pLTV_1$-transformed bacilli (WH-2 and WH-3) on BM2βTNT or BM3βTNT agar plate after application of bacterial (in 8 LB spots) on Day 1 and TNT-DMSO (in 4~8 spots ) on Day 2 for incubation culture at 28°C or 38°C for 14 days.

| Exp. No. | Test Bacilli* | Incub. Temp. | Medium (TNT addded On Day 2) | No. of plate | LB-spot color | Colony and color | No. of colonies ($\mu \pm \sigma_{n-1}$) per spot (per 50 µl) | per plate (per 400 µl) | Colony form. rate**** (per million bacteria) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 168-prot (control) | 28°C | BM2βTNT | 3 | No color | No colony | 0.0 | 0.0 | 0.0 |
| 2 | WH-2** | 28°C | BM2βTNT | 3 | Brown | Brown colonies | 7.1 ±4.8 | 56.8 ±5.6 | 1.42/ million |
| 3 | WH-2 | 28°C | BM3βTNT | 3 | Brown | No colony | 0.0 | 0.0 | 0.0 |
| 4 | WH-2 | 38°C | BM2βTNT | 3 | Brown | Brown colonies | 3.5 ±3.1 | 28.0 ±9.5 | 0.70/ million |
| 5 | WH-2 | 38°C | BM3βTNT | 3 | Brown | Brown colonies | 4.9 ±2.4 | 39.2 ±7.2 | 0.98/ million |
| 6 | MR-1 (control) | 28°C | BM2βTNT | 3 | No color | No colony | 0.0 | 0.0 | 0.0 |
| 7 | WH-3*** | 28°C | BM2βTNT | 3 | Brown | Brown colonies | 8.9 ±9.9 | 70.0 ±7.4 | 3.18/ million |
| 8 | WH-3 | 28°C | BM3βTNT | 3 | Brown | No colony | 0.0 | 0.0 | 0.0 |
| 9 | WH-3 | 38°C | BM2βTNT | 3 | Brown | Brown colonies | 5.4 ±2.7 | 35.0 ±14.5 | 1.59/ million |
| 10 | WH-3 | 38°C | BM3βTNT | 3 | Brown | Brown colonies | 4.8 ±2.9 | 38.4 ±12.8 | 1.75/ million |

* Test bacteria in LBET medium containing at the late stage of stationary phase was frozen in the deep freezer after the addition of 15% glycerol. The frozen stocks were removed from the deep freezer and thawed just before the experiment.

** B.subtilis 168 prototrophic transformed with plasmid $pLTV_1$ was designated as WH-2.

*** MR-1 (B. mycoides ) transformed with plasmid $pLTV_1$ was designated as WH-3.

**** Colony formation rate per million is calculated from the number of colony formation on test medium per that on the TBAB plate with the dilution factor of a million. It is based from the dilution experiment that 1 million dilution of test bacteria WH-2 will form 100 colonies/ml on TBAB plate and that the test bacteria of WH-3 in 1 million times dilution will form 55 colonies per ml on the TBAB plate.