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Characterization of 2,4,6-Trinitrotoluene (TNT)-Metabolizing Bacteria Isolated from TNT-Polluted Soils in the Yamada Green Zone, Kitakyushu, Japan

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Some soils in the Yamada Green Zone, Kitakyushu City, Japan, have been polluted by 2,4,6-trinitrotoluene (TNT), one of explosives. In order to study the biodegradation and behavior of TNT in this zone, we screened the soils for TNT-biodegrading bacteria. We found 6 strains. *Pseudomonas* sp. strains TM15 and TM30, and *Sphingomonas* sp. strain TM22 could biotransform TNT much better than *P. putida* strain TM38, *P. fluorescens* strain TM42, and *Pseudomonas* sp. strain TM55. Their siblings possessing resemblances with these 3 strains were specially isolated from the TNT-contaminated soils in this zone. Six strains converted TNT into reduction products, 2-hydroxylamino-4,6-dinitrotoluene (2HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT). Strain TM38 also produced 2,4-dinitrotoluene (24DNT). TNT has a strong cytotoxicity although its metabolites were slight effectives. The mutagenicity of 2HADNT and 4HADNT was high at the same level or more as that of TNT. The mutagenicity of 2ADNT, 4ADNT and 24DNT remained high, but were less than that of TNT.

Key words: 2,4,6-trinitrotoluene, soil microorganisms, biotransformation, mutagenicity, deformed frogs

1. Introduction

Since the Industrial Revolution, massive amounts of chemicals have been produced and released into the environment around the world[1,17]. Some chemical compounds such as polychlorinated dioxins and polychlorinated biphenyls (PCBs) resist degradation because of their stable and tight structures, and remain in the environment, presenting significant risks of toxicity, mutagenicity and carcinogenicity to animals, especially mammals such as human being[6,11,18,24,27,29,30,38]. Such xenobiotics are degraded primarily by soil microorganisms possessing species and biodegradability of the marvelous of diversities, and partly by ultraviolet light. Thus, understanding the role of microorganisms in these biotransformations is essential for understanding the behavior of these materials in the environment.

The present Yamada Green Zone in Kitakyushu City (at latitude 33° north and longitude 130° east) was once used for packing 2,4,6-trinitrotoluene (TNT) and as an ammunition bunker[15]. Those activities ceased in 1972 and the Yamada Green Zone was declared as the wide-area park in 1995. Since 1995, frogs (*Rana ornativentris*) have been found with superfluous forelegs in this zone. Mating experiments by Kashiwagi et al.[20] revealed that these malformations are heritable. This finding suggests that the deformities are caused by genetic mutations. Several questions thus arise: What caused the decisive and fatal mutations? What are the mutations? How are the deformities caused? Why haven't these deformed frogs disappeared though natural selection? These questions are interesting from the viewpoints of environmental microbiology, genetics, population genetics and embryology. Chemical analysis of the soils in the Yamada Green Zone revealed a high concentration of TNT[21], but only background concentration of polychlorinated dibenzo-p-dioxins, dibenzofurans and PCBs as well as those in other general sites[19]. Therefore, TNT and/or its breakdown products might affect the frog's genes. TNT is one of the toxic chemicals in explosives because of the symmetric location of the nitro groups on the aromatic ring, which limits attacks by enzymes involved in the metabolism of aromatic compounds[14]. Also, TNT generates the frog's chromosomal aberration[21]. Because of this special feature, TNT is persistent in the environment, presenting the risk of the toxicity, mutagenicity and carcinogenicity to animals[34,37].

It is not clear whether soil bacteria in the Yamada Green

Zone convert TNT into metabolites toxic to DNA. We began our research into the biodegradation and dynamics of TNT by the microorganisms present in the Yamada Green Zone by screening soil from that area for TNT-biodegrading bacteria. In this paper, we report on the identification and characterization of some of the strains we isolated, on the biodegradability of TNT by these bacteria, and on the mutagenicities of TNT and its metabolites produced by these strains. We also briefly discuss the possibility of a relationship between the mutagenicities of TNT-degradation compounds and deformed frogs in the Yamada Green Zone.

2. Materials and Methods

2.1. Materials and chemicals

Soil samples were collected from 4 sites contaminated with TNT and 3 clean sites, and then screened for bacteria. As chemical standards we used TNT (a gift of Chugoku Kayaku Co. Ltd., Japan), 4-hydoroxylamino-2,6-dinitrotoluene (4HADNT) (a gift of Dr. Y. Kumagai, Tsukuba University, Japan), 2-hydroxylamino-4,6-dinitrotoluene (2HADNT) (a gift of Dr. R. Spanggord, SRI International, USA), 2-amino-4,6-dinitrotoluene (2ADNT), 4-amino-2,6-dinitrotoluene (4ADNT) (AccuStandard Inc., USA), 2,4-dinitrotoluene (24DNT) and 2,6-dinitrotoluene (26DNT) (Tokyo Kasei Kogyo Co. Ltd., Japan). All chemicals were of the highest purity commercially available. TNT solution (100 mg/L) was autoclaved (110°C , 10 min).

2.2. Screening and identification of TNT-metabolizing bacteria

M8 medium was identical to the M9 medium[25], except that NH_4Cl was omitted. TNT solution (100 mL) with M8 minimal medium containing TNT (100 mg/L) and acetate (10 mM) as nitrogen and carbon sources was mixed with 20 g of soil, and then the mixture was aerobically incubated at 30 °C in the dark with shaking (120 rpm). After 1-week incubation, serial dilutions were spread on M8 minimal medium agar plates with TNT and acetate. Colonies were isolated and characterized using standard procedures including the NF-18 Quick Identification Kit (Nissui, Japan) and API20NE bacterial identification system (BioMerieux, Japan), and by homology search of 16S ribosomal RNA genes[22,23]. Independent 200 clones from 1 soil sample were isolated and examined. Sequences of 16S rDNA were compared to the known DNA sequences in the composite nonredundant database (including GenBank, EMBL, DDBJ and PDB) by using the BLAST search program.

2.3. Culture conditions

All isolated bacteria were aerobically grown until the late logarithmic phase in Luria-Bertani (LB) broth (10 g Bactotryptone, 5 g Bacto yeast extract and 5 g sodium chloride/L-distilled water) containing 100 mg/L of TNT at 30°C in the dark. Cells were washed twice with M9 buffer, and then were resuspended in 0.5 mL of the same buffer. Cell suspensions were mixed into the TNT solution.

2.4. TNT biodegradation by isolated bacteria

Cells (about 5×10^6 cells/mL) were incubated in TNT solution (100 mL) with M8 minimal medium containing TNT (100 mg/L) and acetate (10 mM) at 30°C in the dark with shaking (120 rpm). Percentage biotransformation was calculated from the disappearance of TNT. We measured the degrading of TNT and the production of nitrite from TNT to see whether these bacteria could utilize TNT as carbon and nitrogen sources. Cells were removed by centrifugation (5000×*g*, 10 min). The concentration of TNT was determined by measuring the absorbance at 447 nm of the supernatant (1.5 mL) supplemented with 240 µL 1 M NaOH[26]. Biodegradation of the aromatic ring of TNT was calculated from the disappearance of the aromatic ring as measured by absorbance at 230–280 nm. Nitrite was determined by the method of Snell et al.[31]. The absorbance was measured with a UV/VIS Spectrophotometer V-530 (Jasco, Japan).

2.5. Identification of TNT metabolites

The cells were aerobically grown for different periods (indicated times in Table 2) at 30°C and were removed by centrifugation at 5000×*g* for 10 min. The culture fluid was extracted 3 times with 100 mL ethyl ether (pH 7.0 or 2.0), then the extracts were dried over anhydrous sodium sulfate, and excess solvent was removed by rotary evaporation under pressure at 30°C. Gas chromatography-mass spectrometry (GC-MS) analyses of these samples were carried out with a HP6890 Series GC system/5973 Mass selective Detector (Hewlett Packard, USA). TNT metabolites were identified by comparison of retention times and mass spectra between samples and standard chemicals. Also, detection of 2HADNT and 4HADNT were carried out by high-performance liquid chromatography (HPLC). HPLC analyses were performed on an Inertsil ODS-2 column (GL Sciences Inc., Japan) with acetonitrile-water (40 : 60) as the mobile phase, with a flow rate of 0.4 mL/min. These chemicals were detected at 254 nm with a Shimazu SPD-10AVP UV-VIS detector (Japan).

2.6. Cytotoxicity and mutagenicity

Cytotoxicity was assayed as follows. Frozen stock of *Escherichia coli* K-12 strain AB1157[2] was streaked onto LB plates and grown overnight at 37°C. A single colony was then suspended in 1.5 mL M8 buffer. Cell suspension (10 µL) and LB medium (5 mL) containing TNT, 2ADNT, 4ADNT, 24DNT, 2HADNT or 4HADNT (100 parts LB medium to 1 part chemical in dimethylsulfoxide (DMSO); chemicals tested at 0, 55, 110, 220 and 440 µM) were mixed and incubated for 12 h at 37°C. Serial dilutions were spread on LB agar plates. These plates were incubated for 24 h at 37°C. Survival rate was calculated as the ratio of viable cells in each concentration of TNT, 2ADNT, 4ADNT, 24DNT, 2HADNT and 4HADNT to those in control. We also used

the *umu* test with bioluminescent bacteria (*Salmonella typhimurium* strain TA1535/pTL210, harboring the transcriptional fusion between the *S. typhimurium umuC* gene and the *lux* photoprotein genes from *Vibrio fischeri* (a gift of Toyota Central R&D Laboratories, Japan) to examine the mutagenicity of TNT and its metabolites[33,35]. In this strain, expression of the *lux* operon is under the control of the *umuC* promoter, from which transcription is induced by DNA-damaging agents such as TNT. The intensity was calculated from the integration value per second per well at maximum luminescence with a JNR AB-2100 Luminescencer (ATTO, Japan).

3. Results

3.1. Dynamics of TNT-metabolizing bacteria

TNT-biodegrading clones were found in both TNT-polluted and unpolluted soils. However, all clones obtained from unpolluted soils produced smaller colonies on M8 minimal medium with TNT and acetate than those from polluted soils (data not shown). All pure clones were Gram-negative, non-glucose-fermenting rod bacteria. Characterization using the NF-18 and API20NE systems and homology searches of 16S ribosomal RNA genes classified these clones into 5 strains in the genus *Pseudomonas* and 1 strain in *Sphingomonas*. Four strains, designated as *Pseudomonas* sp. strain TM15, TM30 and TM55, and *Sphingomonas* sp. strain TM22, may be new species, judging from the 16S rDNA results. The DNA homologies of TM15, TM22, TM30 and TM55 were 95.4% in *Pseudomonas olevorans*, 91.8% in *Sphingomonas subractica*, 96.2% in *Pseudomonas putida* and 95.9% in *Pseudomonas aeruginosa*, respectively. The remaining strains were designated as *P. putida* strain TM38 and *Pseudomonas fluorescens* strain TM42. *Pseudomonas* sp. strains TM15 and TM30, and *Sphingomonas* sp. strain TM22 were able to efficiently biotransform TNT (Fig. 1). Isolates with biochemical characteristics and 16S rDNA sequences similar to those of TM15, TM22 and TM30, were also obtained from some TNT-polluted soils (Site A, B, C and D indicated in Table 1)

Table 1. TNT biotransforming bacteria isolated from the soils of the Yamada Green Zone and their composition.

Site	TNT concentration (μg/kg dry-soil)	Strain	Isolation rate[a]
A	120	*Pseudomonas* sp. TM15	0.27
		Sphingomonas sp. TM22	0.25
		Pseudomonas sp. TM30	0.21
		Pseudomonas sp. TM55	0.12
		P. putida TM38	0.15
B	67	*Pseudomonas* sp. TM15 like[b]	0.20
		Sphingomonas sp. TM22 like	0.24
		Pseudomonas sp. TM30 like	0.14
		Pseudomonas sp. TM55 like	0.13
		P. putida TM38 like	0.13
		P. fluorescens TM42	0.16
C	13	*Pseudomonas* sp. TM15 like	0.18
		Sphingomonas sp. TM22 like	0.31
		Pseudomonas sp. TM30 like	0.31
		Pseudomonas sp. TM55 like	0.09
		P. putida TM38 like	0.11
D	7.5	*Sphingomonas* sp. TM22 like	0.28
		Pseudomonas sp. TM30 like	0.31
		Pseudomonas sp. TM55 like	0.19
		P. putida TM38 like	0.22
E	N.D.[c]	*Sphingomonas* sp. TM22 like	0.07
		Pseudomonas sp. TM55 like	0.43
		P. putida TM38 like	0.5
F	N.D.	*Pseudomonas* sp. TM55 like	0.46
		P. putida TM38 like	0.54
G	N.D.	*Pseudomonas* sp. TM55 like	0.40
		P. putida TM38 like	0.36
		P. fluorescens TM42 like	0.24

[a] Two hundreds of independent clones were tested as described in Material and Methods from each soil samples. Isolation rate indicates the ratio of isolated bacteria from each soil.
[b] These strains have the sample characterization as beacteria isolated from soil A.
[c] Not detected.

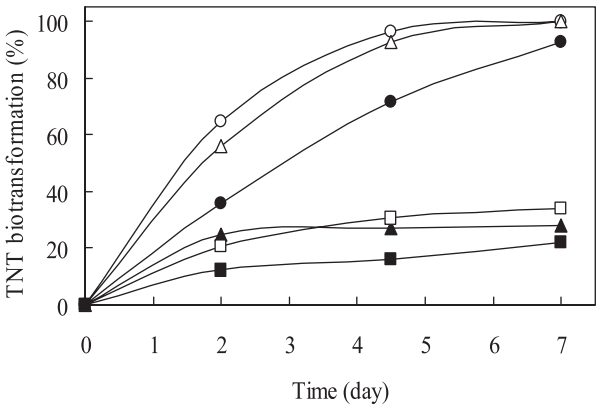

Fig. 1. Biotransformation of TNT by the bacteria isolated from the TNT-polluted soils. The cells were incubated in TNT solution (100 mL) with M8 minimal medium containing TNT (100 mg/L) and acetate (10 mM) at 30°C in the dark with shaking (120 rpm). ○, *Pseudomonas* sp. TM15. △, *Sphingomonas* sp. TM22. ●, *Pseudomonas* sp. TM30. ▲, *Pseudomonas* sp. TM55. □, *P. putida* TM38. ■, *P. fluorescens* TM42.

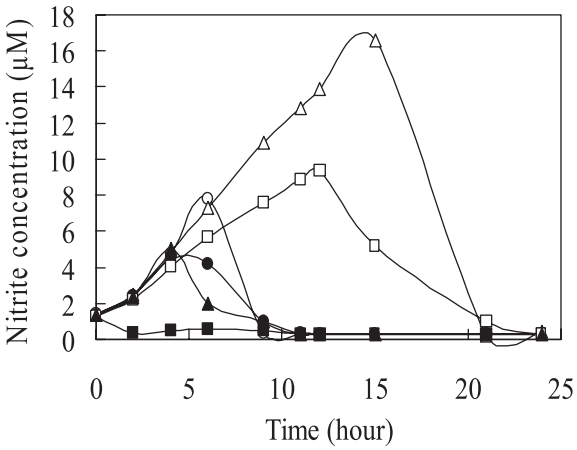

Fig. 2. Release of nitrite by the bacteria isolated from the TNT-polluted soils. The cells were incubated in TNT solution (100 mL) with M8 minimal medium containing TNT (100 mg/L) and acetate (10 mM) at 30°C in the dark with shaking (120 rpm). ○, *Pseudomonas* sp. TM15. △, *Sphingomonas* sp. TM22. ●, *Pseudomonas* sp. TM30. ▲, *Pseudomonas* sp. TM55. □, *P. putida* TM38. ■, *P. fluorescens* TM42.

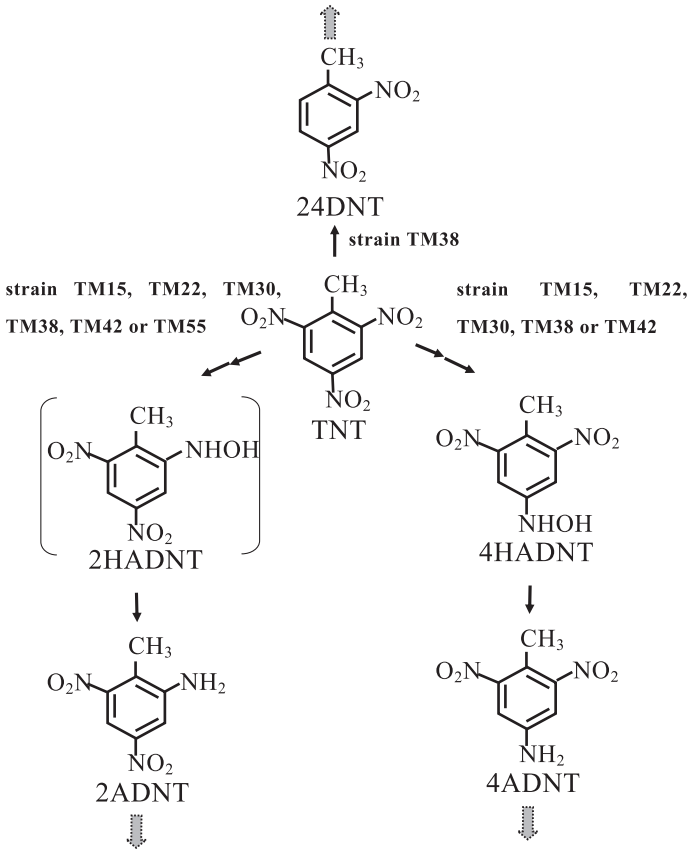

Fig. 3. Products resulting from TNT metabolism by isolated bacteria. 2ADNT, 4ADNT and 24DNT were identified by GC-MS analyses. 2HADNT and 4HADNT are detected by HPLC analyses. TNT, 2,4,6-trinitrotoluene. 2ADNT, 2-amino-4,6-dinitrotoluene. 4ADNT, 4-amino-2,6-dinitrotoluene. 24DNT, 2,4-dinitrotoluene. 2HADNT, 2-hydroxylamino-4, 6-dinitrotoluene. 4HADNT, 4-hydroxylamino-2, 6-dinitrotoluene.

Table 2. Time course of TNT degradation to 2ADNT, 4ADNT and 24DNT by isolated bacteria.

strain	chemicals[a]	Reaction time (day)			
		0	1	3	7
TM15	TNT	440.0[b]	164.4	25.2	1.8
	2ADNT	N.D.[c]	7.5	1.8	0.2
	4ADNT	N.D.	9.3	2.9	0.2
	24DNT	N.D.	N.D.	N.D.	N.D.
TM22	TNT	441.3	173.9	37.8	2.4
	2ADNT	N.D.	15.7	2.4	0.4
	4ADNT	N.D.	20.6	2.9	0.7
	24DNT	N.D.	N.D.	N.D.	N.D.
TM30	TNT	440.8	242.2	121.1	38.0
	2ADNT	N.D.	21.2	39.3	18.3
	4ADNT	N.D.	21.4	39.8	20.1
	24DNT	N.D.	N.D.	N.D.	N.D.
TM38	TNT	439.6	373.7	329.3	327.3
	2ADNT	N.D.	14.9	34.5	16.8
	4ADNT	N.D.	6.2	11.7	6.0
	24DNT	N.D.	6.0	2.5	1.1
TM42	TNT	437.1	415.2	393.6	358.5
	2ADNT	N.D.	7.5	16.1	11.93
	4ADNT	N.D.	2.0	3.3	1.5
	24DNT	N.D.	N.D.	N.D.	N.D.
TM55	TNT	448.8	394.9	353.8	353.4
	2ADNT	N.D.	16.7	39.1	39.9
	4ADNT	N.D.	N.D.	N.D.	N.D.
	24DNT	N.D.	N.D.	N.D.	N.D.

[a] TNT, 2ADNT, 4ADNT and 24DNT indicated the compounds of 2,4,6-trinitrotoluene, 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene and 2,4-dinitrotoluene, respectively.
[b] μM
[c] Not detected.

while isolates similar to the remaining strains were obtained from polluted and unpolluted both soils. Nitrite was transiently accumulated and decreased in all bacterial cultures except that of strain TM42 (Fig. 2), indicating that these bacteria can release nitrite from TNT or the TNT metabolites and utilize it as a sole nitrogen source.

3.2. TNT biotransformation by isolated bacteria

HPLC analysis detected 2HADNT and 4HADNT, and GC/MS analysis identified 2ADNT, 4ADNT and 24DNT as the metabolites from TNT. All strains except *Pseudomonas* sp. strain TM55 biotransformed TNT into 2HADNT, 4HADNT, 2ADNT and 4ADNT. TM55 accumulated only 2ADNT (Fig. 3). *P. putida* strain TM38 also converted TNT into 24DNT. The accumulation of 2,6-dinitrotoluene was not observed in all bacteria. The production of these compounds by each strain is shown in Table 2. 2ADNT and/or 4ADNT in the cultures of 6 strains and 24DNT in the culture of TM38 were transiently accumulated, and then gradually decreased with time. Interestingly, strains TM38, TM42 and TM55, which degraded TNT slowly, apparently biotransformed TNT into 2ADNT in preference to 4ADNT, which was predominant in 3 strains that biodegraded TNT faster than TM38, TM42 and TM55.

3.3. Cytotoxicity and mutagenicity of TNT and its initial metabolites

We tested whether the 2ADNT, 4ADNT, 24DNT, 2HADNT and 4HADNT produced from TNT by the soil microorganisms are cytotoxic to *E. coli* K-12 strain AB1157. Increasing concentrations of TNT dramatically decreased survival, whereas 2ADNT, 4ADNT, 24DNT, 2HADNT and 4HADNT had only a slight effect (Fig. 4A). The mutagenicities of 2HADNT and 4HADNT were similar to or higher than that of TNT. The mutagenicities of 2ADNT, 4ADNT and 24DNT were lower than that of TNT, although still substantial (Fig. 4B). In the results of TNT mutagenicity, luminescence disappeared above 71 pmol of TNT, but remained in the presence of the other chemicals. The reason is probably that the luminescent bacteria were killed by a high concentration of TNT, which has a strong cytotoxicity.

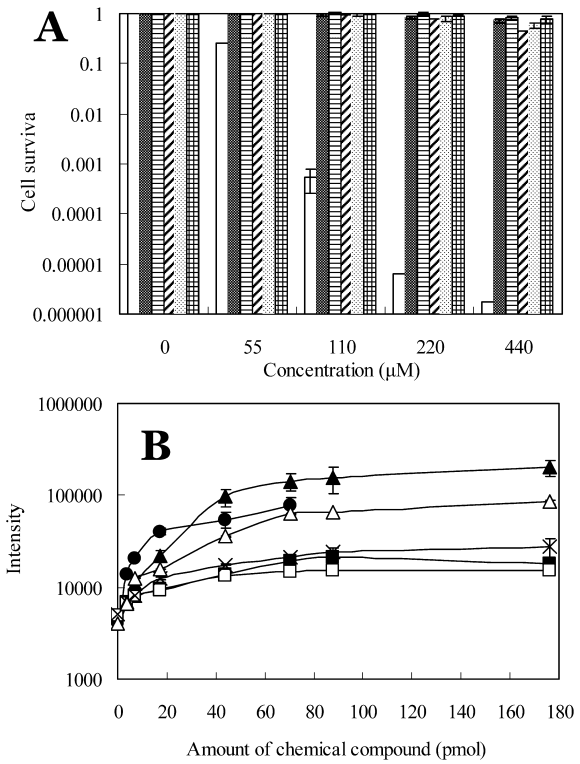

Fig. 4. Cytotoxicity and mutagenicity of TNT and its metabolites, 2HADNT, 4HADNT, 2ADNT, 4ADNT and 24DNT, produced by isolated bacteria. (A) Survival of *E. coli* cells exposed to TNT (white bars), 2ADNT (black bars), 4ADNT (banded bars), 24DNT (hatched bars), 2HADNT (gray bar) and 4HADNT (grille bars). Data are averages of 5 independent experiments; error bars indicate the standard errors of the means. (B)Assay of mutagenicity of TNT (●), 2HADNT (▲), 4HADNT (△), 2ADNT (■), 4ADNT (□) and 24DNT (×).

4. Discussion

We isolated TNT-biodegrading bacteria from the soils in the Yamada Green Zone, where abnormal frogs have appeared and where TNT has been detected. These bacteria converted TNT into 2HADNT, 4HADNT, 2ADNT, 4ADNT and 24DNT (Fig. 3), in agreement with the results of previously studies[9,14,32]. These bacteria seem to be involved in the degradation of TNT in the Yamada Green Zone. 2ADNT and 4ADNT are produced from TNT by six-electron reduction of its nitro group. Barrow et al.[4] suggested that the nitro group in the *para* position is much more easily reduced than those in the *ortho* positions of TNT because of an electron-donor effect of the methyl group (namely, accumulation of 4ADNT is expected to be higher than that of 2ADNT). Strains TM15, TM22 and TM30, which were able to efficiently degrade TNT, preferentially accumulated 4ADNT, in contrast to strains TM38, TM42 and TM55, which preferentially accumulated 2ADNT (Table 2). These results may be due to differences in substrate specificity of degrading enzymes and/or in the subsequent biodegradability of 2ADNT and 4ADNT. Strain TM38 also converted TNT to 24DNT through the elimination of a nitro group (Fig. 3). This result resembles the TNT biotransformation pathway reported by Duque et al.[8].

As shown in Fig. 4-B, 2HADNT and 4HADNT are strong mutagens as well as TNT and they have reported in some previous reports[3,5,28]. Homma-Takeda et al. reported that these compounds caused oxidative DNA damage (formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine) in the presence of NADH and Cu(II)[16]. Also, partially reduced forms of TNT react among themselves under aerobic conditions to form recalcitrant tetranitro-azoxytoluenes[12], which cause a higher rate of mutations than does TNT[10]. The mutagenicities of 2ADNT and 4ADNT were less than that of TNT although still high in our results. This result is consistent with previous reports[7,10,34]. 2ADNT and 4ADNT are more stable in the environment and more mobile, posing a potential threat to drinking water supplies, human health and the environment[13]. On the other hand, cytotoxicity of 2HADNT, 4HADNT, 2ADNT, 4ADNT and 24DNT was slight compared to that of TNT (Fig. 4A). Kashiwagi et al.[20] and Kitakyushu City Agency[21] suggested that the frog's deformities were caused by genetic mutations induced by some factors. The results of their mating experiment of four patterns (normal males×normal females, normal males× abnormal females, abnormal males×normal females, and abnormal males×abnormal females) demonstrated that only abnormal offsprings were born from deformed males. Namely, mutations leading to deformities are transferred to descendants by the sperm. They assumed that the mutations were induced in the frog's sperm *in vivo* or fertilized eggs in the environment. Because sperm cells have low activities of DNA repair enzymes[36], mutations are expected to be easily fixed in sperm cells. In this paper, we revealed that TNT and its metabolites have the DNA damaging activities; therefore, as one of possibilities, these compounds may relate to generating the frogs with deformities in the Yamada Green Zone. Developed studies on how these metabolites have genotoxic effects for frogs or their sperm cells will be required. Also, we will need to research detail distribution analyses (dynamics) for accumulation of TNT and its metabolites in the Yamada Green Zone.

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