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Characterization of *Pseudomonas* sp. HK-6 cells responding to explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine)

Abstract The cellular responses of *Pseudomonas* sp. HK-6 to explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) have been extensively analyzed in this study. The stress shock proteins, which might contribute to enhancing the cellular resistance to the cytotoxic effect of RDX, were induced at different concentrations of RDX used as a substrate for cell culture of *Pseudomonas* sp. HK-6. The proteins were identified as 70-kDa DnaK and 60-kDa GroEL by SDS-PAGE and Western blot using the anti-DnaK and anti-GroEL monoclonal antibodies. The stress shock proteins induced by RDX were found to increase in proportion to the RDX concentration used for this work. Analysis of membrane fatty acids of strain HK-6 following exposure to RDX showed that the amounts of dominant lipids 16:1 ω7c/15:0 iso 2OH, 16:0 and 18:1 ω7c/ω9t/ω12t decreased substantially or were not detected in the cells exposed to RDX, while amounts of lipids 10:0 iso, 14:1 ω5c/ω5t and 16:10 methyl increased dramatically. Scanning electron microcopy analyses revealed the presence of perforations and irregular rod shapes with wrinkled surfaces for cells treated with 0.135 mM RDX for 12 h, suggesting that RDX has a substantial cytotoxic impact on cells of strain HK-6.

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Introduction

The explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a nitroaromatic compound which makes up a great proportion of military waste, giving rise to serious environmental problems with trinitrotoluene. Previous studies have reported that many microorganisms exhibit various responses to environments persistently polluted with chemicals or toxic agents such as industrial organics, pesticides, alcohols, and phenols as agents of environmental stress (Lupi et al. 1995; Cho et al. 2000; Park et al. 2001). Microorganisms in environments that are heavily contaminated with these chemicals might experience serious damage to the cellular pH gradient, shifts in cellular fatty acid composition, denaturation of some proteins, and ultimately cell death (Warth 1991; Pinkart et al. 1996; Cho et al. 2002). Microbial responses to contaminated environments include shifts of membrane lipids and production of stress shock proteins (SSPs) such as DnaK and GroEL. Recently, the SSPs induced by chemical stresses have been analyzed in several bacteria such as *Acinetobacter radioresistens* and *Acinetobacter lwoffii* K24 using proteome analysis (Giuffrida et al. 2001; Kahng et al. 2002; Kim et al. 2002). These results suggested that chemicals (e.g., benzoate, aniline, phenol) induced biodegradation-related enzymes as well as other metabolic enzymes such as stimulons. Our recent reports demonstrated the induction of SSPs in *Burkholderia cepacia* YK-2 capable of 2,4-D degradation in response to phenoxyherbicides 2,4-D and 2,4,5-T as environmental contaminants (Cho et al. 2002). However, further information on microbial responses to explosives is required to establish scientific and systematic data. These requirements stimulated our studies on microbial responses to another explosive chemical, RDX, because microbial responses to this explosive have not yet been reported. In addition, induction of SSPs by RDX in microorganisms could be used as a tool in environmental monitoring; synthesis of SSPs may serve as a biological indicator by which the presence of toxic environmental pollutants can be established.

In this study, strain *Pseudomonas* sp. HK-6, an RDX-degrading bacterium, was enriched and screened from soils contaminated with RDX in Korea and identified by the Biolog analysis system. Rate of RDX degradation and survival rates were determined, and associated SSPs induced by strain HK-6 under RDX stress conditions were analyzed by SDS-PAGE and Western blots, together with the shift in total cellular fatty acid composition of strain HK-6. Scanning electron microscopy (SEM) analyses were additionally performed under lethal concentrations of RDX to evaluate the morphological changes that occurred in cell envelope following exposure to RDX.

Materials and methods

Bacterial isolation and culture conditions

Bacterial enrichment cultures capable of utilizing RDX were derived from soil samples that had a previous history of RDX exposure. For RDX degradation, cells were grown in liquid medium composed of 10 mM K_2HPO_4, 5 mM NaH_2PO_4, 1 mM $MgSO_4.7H_2O$, 0.07 mM $CaCl_2.2H_2O$, 0.04 mM $FeCl_3.6H_2O$, 0.0005 mM $MnCl_2.4H_2O$, 0.00035 mM $ZnSO_4.7H_2O$, 5 mM succinate, and 0.045–0.135 mM RDX. From the enriched bacterial mixture, a pure strain, designated HK-6, was isolated based on the ability to degrade RDX, and it was routinely maintained on culture media containing desired concentrations of RDX. Cultures were grown at 30°C and aerated on a rotary shaker (New Brunswick Scientific, Edison, N.J.) at 150 rpm. Every 4 days, 1 ml of the culture was withdrawn and used to determine the cell density (absorbance at 600 nm) by spectrophotometer.

A GN2 MicroPlate (Biolog, Hayward, Calif.) was used to characterize strain HK-6 based on substrate utilization profiling. A single HK-6 colony grown on trypticase soy agar (TSA) was streaked onto Biolog universal growth agar medium containing 5% sheep blood and incubated overnight at 30°C. Cells were suspended in normal saline (0.15%) and inoculated onto the GN2 MicroPlate. After incubation for 20 h, the resulting pattern was read using the Biolog automated Micro-Station instrument.

Analytical methodology

Residual RDX was determined by reverse-phase high performance liquid chromatography (HPLC). The HPLC system consisted of a pump (Shimadzu LC-10A, Japan), an injector fitted with a 20-μl loop, UV detector, and integrator. Analytes were detected by using a commercial Zorbax ODS reverse column (C_{18}, 250 mm × 4.6 mm, particle size 5 μl) and a mobile phase of a mixture of 40% (v/v) acetonitrile and 80% water at a flow rate of 1.0 ml min^{-1}. The samples were centrifuged at 3,500 g for 10 min to sediment the bacterial cells. Aliquots (1 ml) of the supernatants were diluted with HPLC grade water before filtration through a 0.45 μm Gelman Arco LC25 disposable syringe filter. For peak identification, the retention times of the unknown peaks were compared with those of authentic reference compounds. Standard curves were constructed by plotting the peak areas versus known amounts of the authentic standards.

Stress treatment with RDX and survival test

The cells grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) were harvested by centrifugation at 2,000 g for 10 min. These cells were washed three times with 10 mM phosphate buffer (pH 7.0) and then inoculated to approximately 10^8 cells ml^{-1} in 30 ml liquid medium in 100-ml Erlenmeyer flasks containing 0.045 mM, 0.09 mM, or 0.135 mM RDX. The organisms were exposed to RDX in shake flasks at 30°C. After exposure for the optimum period, the viable cells were counted by plating them on LB agar.

Extraction and analysis of total cellular fatty acids

Cells harvested after 24 h of growth on TSA were heated with NaOH–methanol to saponify cellular lipids, and the released fatty acids were methylated by heating with HCl–methanol. Fatty acid methyl esters (FAMEs) were solvent-extracted and analyzed by gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). FAMEs were identified by comparing their retention times and mass spectra with those of authentic standards provided in the MIDI database. To examine fatty acid changes that occurred as a result of exposure to RDX, cells grown on TSA were collected and washed twice in potassium phosphate buffer (pH 7.0). Washed cells were incubated in 0.135 mM RDX in the liquid medium as described above. Cells were exposed to explosive for 24 h, and lipids extracted from these cells were used for fatty acid analysis.

SDS-PAGE

After the organisms were treated with RDX, the cells were collected by centrifugation at 2,000 g and suspended in 10 mM phosphate buffer (pH 7.0). Cells in the phosphate buffer were disrupted by ultrasonification (Fisher M-300, Pittsburgh, Pa.). Prior to SDS-PAGE analysis, the proteins were quantified with a protein assay kit (Sigma, St. Louis, Mo.) according to the manufacturer's instruction. SDS-PAGE of proteins was performed according to the method described by Bollag et al. (1992), with 12% acrylamide for separating gel and 4% acrylamide for stacking gel, with a running buffer (0.025 M Tris, 0.192 glycine, 0.1% SDS, pH 8.3) at 60–90 V for 2.5 h. Gels were stained with a staining solution (0.1% Coomassie brilliant blue R-250, 40% methanol, 7% glacial acetic acid) for 2 h. The gels were destained with solution I (50% methanol, 10% glacial acetic acid) for 1 h, and then with solution II (5% methanol, 7% glacial acetic acid) for 10 h.

Western blotting

RDX-treated cells were analyzed for SSPs by the Western blot technique (Sambrook et al. 1995) with anti-DnaK and anti-GroEL monoclonal antibodies (StressGen Biotechnologies, Victoria, B.C., Canada), which were induced by heat shocking *Escherichia coli* (70 kDa for DnaK and 60 kDa for GroEL). The proteins on the gels separated by SDS-PAGE were transferred to Hybond PVDF membrane (Amersham, Buckinghamshire, UK) with a Semiphor semi-dry transfer unit (Owl Separation Systems, Portmouth, N.H.). The blots were blocked with 0.1% bovine serum albumin for 1 h at 22±2°C. Subsequently, the blots were washed with phosphate-buffered saline (PBS) and incubated with primary antibody diluted 5,000 times in PBS–0.08% Tween 20. The secondary antibody (anti-mouse IgG HRP conjugate, Promega, Madison, Wis.) diluted 5,000 times in PBS–0.08% Tween 20 was applied for 1.5 h, and the blots were washed with PBS–0.08% Tween 20. The immunocomplex was detected with an ECL Western analysis system (Amersham) according to the manufacturer's instruction.

Scanning electron microscopy

Colonies of HK-6 grown on LB agar plates for 24 h were excised as small agar blocks of 0.5 cm^3. The agar blocks containing a colony were then exposed to 0.135 mM RDX in liquid medium for 1 h. The

**Table 1** Physiological and biological characterization of *Pseudomonas* sp. HK-6 using the Biolog analyis system

Physiological and biochemical tests

Water	−	*p*-Hydroxyphenylacetic acid	−
α-Cyclodextrin	−	Itanonic acid	−
Dextrin	−	α-Ketobutyric acid	+
Glycogen	+	α-Ketoglutaric acid	+
Tween 40	+	α-Ketovaleric acid	−
Tween 80	+	D,L-Lactic acid	+
N-acetyl-D-galactosamine	−	Malonic acid	+
N-acetyl-D-glucosamine	−	Propionic acid	+
Adonitol	−	Quinic acid	+
L-Arabinose	−	D-Saccharic acid	+
D-Arabitol	+	Sebacic acid	−
Cellobiose	−	Succimic acid	+
i-Erythritol	−	Bromo succinic acid	+
L-Fructose	−	Succinamic acid	−
L-Fucose	−	Glucuronamide	+
D-Galactose	−	Alaninamide	+
Gentiobiose	−	D-Alanine	+
α-D-Glucose	+	L-Alanine	+
m-Inositol	−	L-Alanylglycine	+
α-D-Lactose	−	L-Asparagine	+
Lactulose	−	L-Aspartic acid	+
Maltose	−	L-Glutamic acid	+
D-Mannitol	−	Glycyl-L-aspartic acid	−
D-Mannose	−	Glycyl-L-glutamic acid	−
D-Melibiose	−	L-Histidine	+
β-Methyl D-glucoside	−	Hydroxy-L-proline	+
Psicose	−	L-Leucine	+
D-Raffinose	−	L-Omithine	−
L-Rhamnose	−	L-Phenylalanine	−
D-Sorbitol	−	L-Proline	+
Sucrose	−	L-Pyroglutamic acid	+
D-Trehalose	−	D-Serine	−
Turanose	−	L-Serine	+
Xylitol	−	L-Threonine	−
Methylpyruvate	−	D,L-Camitine	−
Mono-methylsuccinate	+	γ-Aminobutyric acid	+
Acetic acid	+	Urocanic acid	−
cis-Acomitic acid	+	Inosine	−
Citric acid	+	Uridine	−
Formic acid	+	Thymidine	−
D-Galactonic acid lactone	−	Phenylethylamine	+
D-Galacturonic acid	+	Putrescine	+
D-Luconic acid	+	2-Aminoethanol	+
D-Glucosaminic acid	−	2,3-Butanediol	−
D-Glucuronic acid	+	Glycerol	+
α-Hydroxybutyric acid	−	D,L-α-Glycerolphosphate	−
β-Hydroxybutyric acid	+	Glucose-1-phosphate	−
γ-Hydroxybutyric acid	−	Glucose-6-phosphate	−

colonies treated with the explosive compound were pre-fixed with 2.5% glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.2) for 2 h and the post-fixed with 1% osmium tetroxide in the same buffer, as described by Ng et al. (1985). The fixed cells were dehydrated in a series of increasing ethanol concentrations (30–95%) for 15 min and then in 100% ethanol for 20 min. The cells were treated with absolute isoamyl acetate for 15 min and then air-dried. The cells were coated with gold by using a sputter coater (IB-3, Giko Engineering, Japan) and were then examined with a Hitachi S-2500C scanning electron microscope (Hitachi, Japan).

Results

Bacterial isolation and identification

Strain HK-6 was screened from the RDX-degrading enriched culture for its ability to degrade RDX under aerobic conditions. Strain HK-6 was found to be gram-negative, rod-shaped, catalase-positive, oxidase-positive, motile, phenylalanine deaminase-negative, tryptophanase-negative, urease-negative, and negative for starch utilization, indole formation and H_2S formation. The physiological and biochemical characteristics of strain HK-6 obtained from the Biolog system are listed in Table 1. Analysis of the carbohydrate utilization profiles based on the GN2 MicroPlate and analysis of the fatty acid
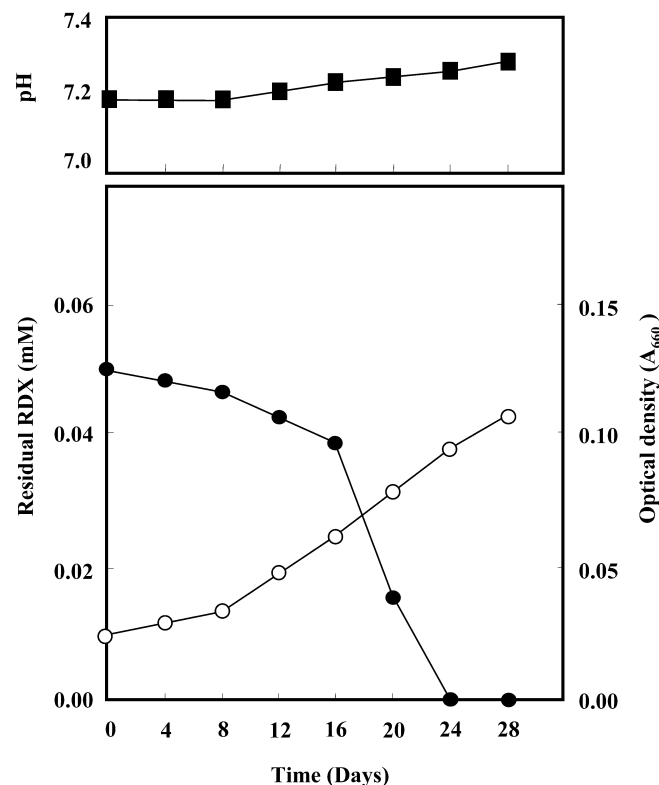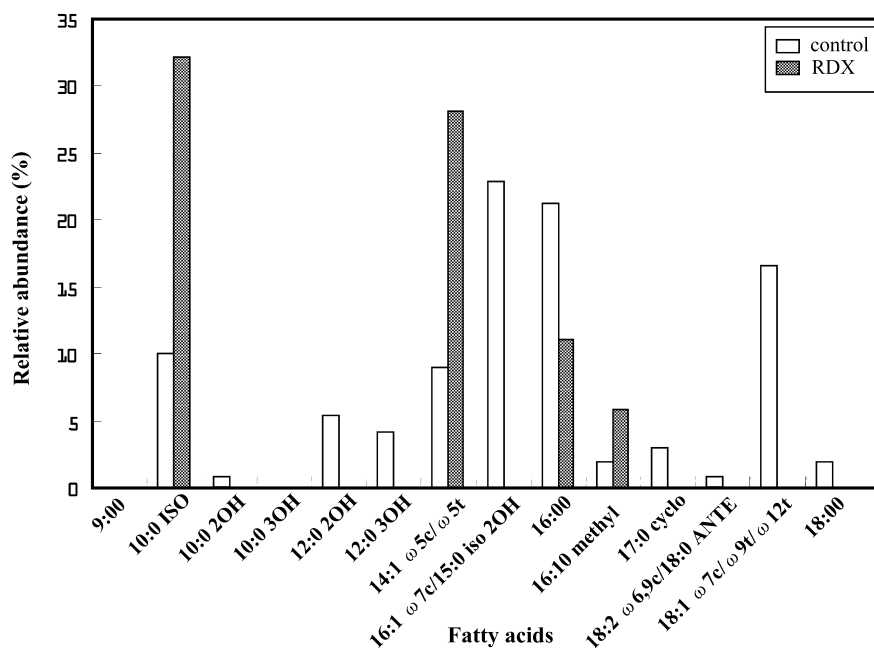

Fig. 1 Cell growth of *Pseudomonas* sp. HK-6 (○) and hexahydro-1,3,5-trinitro-1,3,5-triazine (*RDX*) degradation (●). Growth rate was measured as optical density at 660 nm and rate of RDX degradation was determined every 4 days throughout 24 days incubation by reverse phase HPLC along with pH (■). RDX for this experiment was initially supplied at a concentration of 0.045 mM
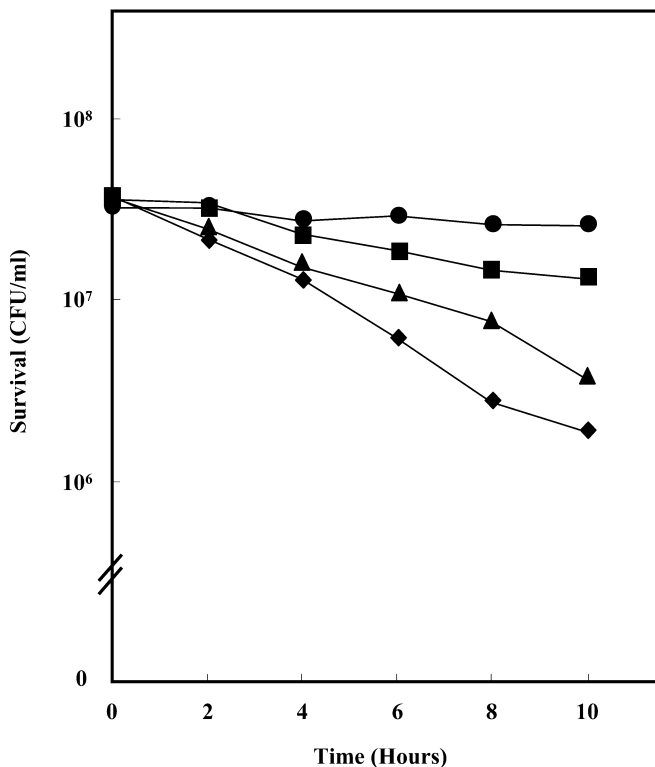
Fig. 2 Survival rate of *Pseudomonas* sp. HK-6 cells following exposure to RDX. HK-6 cells were maintained at the concentrations of 0 mM (●), 0.045 mM (■), 0.09 mM (▲), and 0.135 mM (♦) RDX. At intervals, the numbers of colonies (*CFU/ml*) were measured

composition by GC-MS also placed HK-6 as a *Pseudomonas* species with a confidence of over 97%. On the basis of the results, the isolate could be assigned and designated as *Pseudomonas* sp. HK-6.

Bacterial growth and RDX degradation

Strain HK-6 was initially tested for the degradation of RDX in 250-ml Erlenmeyer flasks under aerobic conditions. HK-6 was able to grow with up to 0.045 mM RDX, which was the highest concentration tested. Changes in the optical density at 660 nm associated with cell growth and the degradation of RDX are shown in Fig. 1. HK-6 cultures completely degraded 0.045 mM RDX within 24 days of incubation. However, optical density of grown cells at 660 nm was quite low (below 0.15). HPLC monitoring of residual RDX from the collected aliquots revealed that metabolic intermediates were not detected during RDX degradation in the culture.

Survival of HK-6 under RDX stress

The survival rate of *Pseudomonas* sp. HK-6 was examined during 0–10 h of incubation in liquid medium containing 0–0.135 mM RDX, and the results are shown in Fig. 2. The survival rate of the strain HK-6 was not greatly affected by the explosive at lower concentrations, but were gradually decreased depending on the increasing RDX concentrations in the media.

Shift in cellular fatty acid composition for RDX-exposed cells

The total cellular fatty acids of strain HK-6 comprised 11 C-even and 2 C-odd fatty acids (fatty acids <0.2% in abundance were not considered in this calculation). The dominant lipids 16:1 ω7c/15:0 iso 2OH, 16:0 and 18:1 ω7c/ω9t/ω12t made up 23%, 21%, and 16% of total cellular fatty acids for cells grown on complex medium

Fig. 3 Fatty acids profiles of *Pseudomonas* sp. HK-6 analyzed by gas chromatography with flame ionization detection and gas chromatography with mass spectrometry when grown on trypticase soya agar (*control*) or RDX. Cells grown on 0.135 mM of RDX for 24 h were used for extraction of total cellular fatty acids, and the lipids were identified based on the retention of authentic references

Fig. 4A–C Induction of stress shock proteins (SSPs) in *Pseudomonas* sp. HK-6 treated with different RDX concentrations for 8 h. The SSPs were analyzed by SDS-PAGE (**A**), and Western blot with **B** anti-DnaK and **C** anti-GroEL monoclonal antibodies
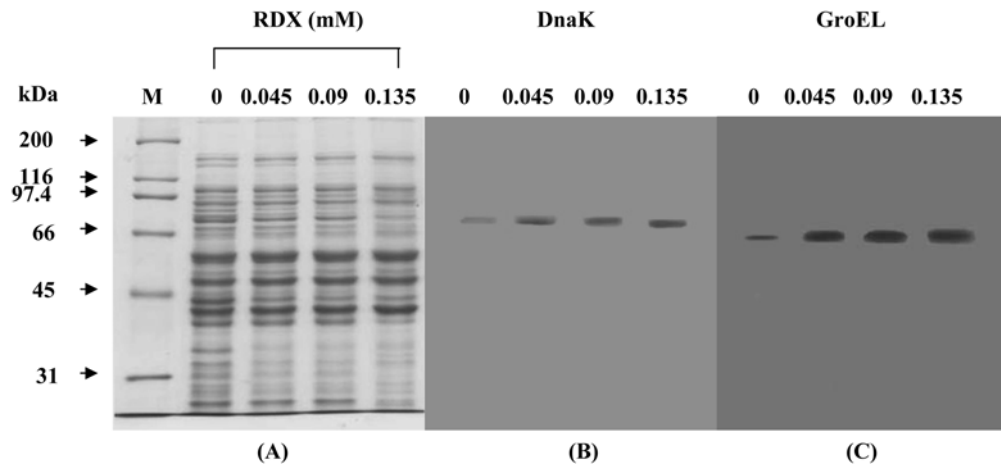
(TSA), respectively (Fig. 3). However, levels of these lipids decreased substantially or were not detected in the cells exposed to RDX. Lipids 10:0 iso and 14:1 ω5c/ω5t, which were 10% and 9% of the total cellular fatty acids for TSA-grown cells, showed dramatic increases to 33% and 28% when cells were exposed to RDX (Fig. 3). Lipid 16:10 methyl that made up 2% of total cellular fatty acids for TSA-grown cells also increased above 5%. In addition to this, lipids 9:00, 10:0 2OH, 10:0 3OH, 12:0 2OH, 17:0 cyclo, 18:2 ω6, 9c/18:0 ANTE, 18:1 ω7c/ω9t/ω12t, and 18:00, which were detected for cells grown on TSA, were no longer detected for the cells grown on RDX.

Production of SSPs

Pseudomonas sp. HK-6 cells treated under sublethal conditions of RDX were examined for the production of DnaK and GroEL SSPs (Table 2). Both DnaK and GroEL proteins began to appear in the cells treated with 0.045–0.135 mM RDX for 4–12 h. However, no GroEL protein was induced in the cells treated with the RDX concentrations tested for 14 h or longer. The DnaK and GroEL SSPs were detected by SDS-PAGE and Western blot with anti-DnaK and anti-GroEL monoclonal antibodies. The profiles of total proteins are shown in Fig. 4A. DnaK and GroEL SSPs produced with RDX treatment are shown in Fig. 4B and C, respectively. The amounts of SSPs induced were proportional to the RDX concentrations exposed to HK-6 cells. Also, results from this study demonstrated that strain HK-6 responded to this stress by production of these proteins, two of which were the 70-kDa DnaK and 60-kDa GroEL proteins.

Effect of the stresses on cellular morphology

The morphological changes of *Pseudomonas* sp. HK-6 cells were examined by exposure to lethal conditions of RDX, which caused a significant decrease in their viability. As seen in the scanning electron micrographs, normal cells grown in complex medium (TSA) in the

Table 2 Stress shock proteins (*SSPs*) produced in *Pseudomonas* sp. HK-6 treated with hexahydro-1,3,5-trinitro-1,3,5-triazine (*RDX*) at different concentrations. + Production, – no production

Treatment period (hours)	SSPs	Induction of SSPs by RDX		
		0.045 mM	0.09 mM	0.135 mM
2	DnaK	–	+/–	+/–
	GroEL	+/–	+	+
4	DnaK	+	+	+
	GroEL	+	+	+
6	DnaK	+	+	+
	GroEL	+	+	+
8	DnaK	+	+	+
	GroEL	+	+	+
10	DnaK	+	+	+
	GroEL	+	+	+
12	DnaK	+	+	+
	GroEL	+	+	+
14	DnaK	–	+/–	+
	GroEL	–	+/–	+/–
16	DnaK	–	–	+/–
	GroEL	–	–	–
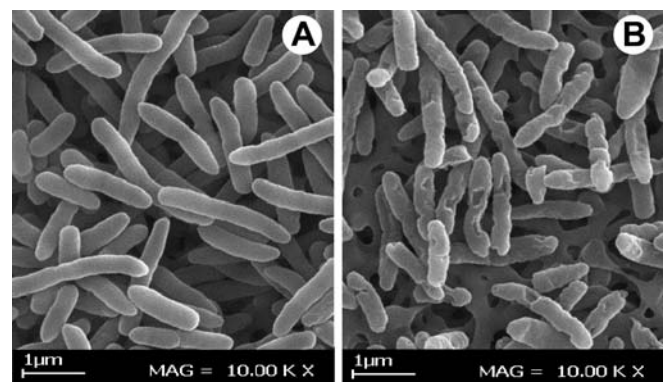
Fig. 5A, B Scanning electron micrographs of *Pseudomonas* sp. HK-6 treated with RDX. **A** Untreated cells, **B** cells treated with 0.135 mM RDX for 12 h

absence of the explosive exhibit a typical rod shape with smooth surface (Fig. 5A). However, the cells treated with 0.135 mM RDX for 12 h showed several destructive openings on the cell envelope, as well as a preponderance of irregular rod forms with wrinkled surfaces. (Fig. 5B).

Discussion

A pure strain HK-6 was isolated from the highly active consortium obtained from the RDX contaminated site. Based on the results of Biolog tests and fatty acid composition by GC-MS, strain HK-6 was placed into *Pseudomonas* species with confidence over 97%, and it was designated as *Pseudomonas* sp. HK-6. The present work focused only on RDX degradation and cellular responses of HK-6 induced by RDX.

Pseudomonas sp. HK-6 was capable of utilizing RDX as a carbon and energy source when it was used at lower concentration of 0.045 mM, so it was considered that the survival of this strain was not affected by the explosive RDX at lower concentrations (Fig. 1). Notably, maximum cell density at 660 nm after 24 days of incubation in RDX medium was below 0.15, which was unexpectedly quite low, suggesting slow rate of RDX transformation by HK-6 cells. This might result from low availability or toxic effects of intermediates produced from RDX degradation to HK-6 cells, warranting further intensive study. When cells were grown at over 0.135 mM concentrations of RDX, there was a pause in cell growth as shown in Fig. 2, resulting in a substantial reduction in the rate of RDX degradation. It was assumed that HK-6 cells might suffer from a considerable toxic effect by high concentrations of over 0.135 mM RDX, despite their ability to utilize RDX. This finding was consistent with previous reports that the survival rates of the organisms gradually decreased as a function of treatment time, and the rates were proportional to the concentration of toxic chemicals (Park et al. 2001; Cho et al. 2002).

Exposure of HK-6 cells to RDX resulted in changes of the total cellular fatty acid composition (Fig. 3). Notably, levels of lipids 10:0 iso, 14:1 ω5c/ω5 t, and 16:10 methyl increased dramatically to 33% (approximately 3.3 times those of non-exposed cells), 28% (3.5 times), and over 5% (2.5 times) for cells exposed to RDX, respectively (Fig. 3). It was considered that changes of these fatty acids in response to RDX might affect cells' tolerance for survival, or enhance cells' ability to utilize the substrate. This finding was consistent with our previous study that several *cis*-unsaturated fatty acids in *Burkholderia* sp. HY1 increased in response to aniline, along with increase of some *trans*-fatty acids (Kahng et al. 2000). This fact suggested lipids 10:0 iso and ω5c/ω14:1 16:10 methyl might play a key role in cells' tolerance or adaptation to RDX, warranting further intensive study.

Pseudomonas sp. HK-6 was able to produce 70-kDa DnaK and 60-kDa GroEL in the presence of sublethal concentrations of RDX as shown in Fig. 4. Western blot analysis revealed clearly that the SSPs were produced even in the absence of RDX, providing evidence that both SSPs always exit in the cell at the basal level (Martin and Hartl 1997; Passow et al. 1997; Ranson et al. 1998). This result was consistent with other reports that most stress genes are expressed in the absence of stress, namely, under normal physiological conditions. In the study of SSP production in *Pseudomonas* sp. DJ-12 to various sublethal environmental stresses (e.g., aromatic hydrocarbons, ethanol, heat), Park et al. (2001) reported that SSPs including DnaK and GroEL were induced in the cells by treatment with heat or chemical stress under sublethal conditions; the organisms also exhibited a certain degree of survival tolerance to the lethal levels of the same stress. Blom et al. (1992) reported that 13–39 stress proteins were induced in *E. coli* when a growing batch culture of the organism was exposed to nine different pollutants. In general, in our study, DnaK and GroEL were produced in increasing amounts in all cells that were treated with respective sublethal stresses, as shown in Fig. 4.

SEM analysis demonstrated that the cells treated with 0.135 mM RDX had some disruptive openings on the cell envelopes and irregular rod forms with wrinkled surfaces were formed (Fig. 5). Ramos et al. (1995) and Skkema et al. (1995) reported that the aromatic hydrocarbons at high concentrations conferred toxic effect to the cells due to the disruption of membrane component, and this led to cell death. *Burkholderia cepacia* YK-2 treated with lethal concentrations of phenoxyherbicides 2,4-D or 2,4,5-T morphologically changed the cells, which developed a rippled cell surface with irregularly shaped and significant alteration (Cho et al. 2002). In this respect, data obtained from SEM analysis for strain HK-6 were consistent with cellular responses against aromatic compounds found in other bacteria.

Although RDX is commonly used by the military as a powerful explosive, it is evident that the toxicity of this compound seriously affects the survival of soil microorganisms in the ecosystem. However, the possible lethal impact of a compound such as RDX against soil microorganisms in an ecosystem where this compound was exposed indiscriminately has never been evaluated. To our knowledge, this is the first report on the cellular responses of soil microorganisms to RDX as an environmental stressor. Therefore, further work, including reverse molecular genetics, will stimulate studies on the elucidation of SSPs and their corresponding genes in strain HK-6 which degrades RDX, providing good information of microbial response to the explosive RDX.

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