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Induction of Stress Shock Proteins DnaK and GroEL by Phenoxyherbicide 2,4-D in *Burkholderia* sp. YK-2 Isolated from Rice Field

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Abstract. The purpose of this work was to investigate the induction of stress shock proteins in *Burkholderia* sp. YK-2 in response to the phenoxyherbicide 2,4-dichlorophenoxyacetic acid (2,4-D). The stress shock proteins, which contribute to the resistance of the cytotoxic effect of 2,4-D, were induced at different 2,4-D concentrations in exponentially growing cultures of *Burkholderia* sp. YK-2. This response involved the induction of a 43-kDa DnaK and 41-kDa GroEL proteins, characterized by SDS-PAGE and Western blot by use of the anti-DnaK and anti-GroEL monoclonal antibodies. The total stress shock proteins were analyzed by 2-D PAGE. Survival of *Burkholderia* sp. YK-2 with time in the presence of different concentrations of 2,4-D was monitored, and viable counts paralleled the induction of the stress shock proteins in this strain.

Exposure of indigenous microorganisms to physical, chemical, and biological agents induces the synthesis of a set of proteins referred to as stress shock proteins (SSPs). Such agents include elevated temperature [1, 8], nutrient limitation or starvation [7], UV light, viral infection, heavy metals [22], and inhibitory or toxic organic chemicals [3]. The most widely studied and the best characterized response in bacteria is the heat shock response [5, 10].

A large number of chemical agents, including the BTEX group (benzene-toluene-ethyl benzene-xylene), 2,4-dinitrophenol, 2,4-dichloroaniline, pentachlorophenol, and trichloroethylene, were reported to induce SSPs [2, 3, 6, 22]. Chemicals have been shown to stimulate the synthesis of SSPs in *Escherichia coli* [3, 6, 12] and *Pseudomonas putida* [9, 14, 17, 18, 24], and some of these responses are chemical specific. Thus, the precise cellular response to a specific stress is characteristic to the chemical.

The phenoxyherbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is a growth-regulating compound. This auxin is selectively toxic to most annual and perennial dicotyledonous species and relatively toxic to monocotylodeonous plants [11, 13, 20]. 2,4-D has numerous applications in a wide variety of situations, including weed control in residential golf courses and lawns, gardens, cereal crops, and pastures, as well as defoliants in forestry and military operations [21]. Although 2,4-D is a widely used herbicide, little is known about the 2,4-D-induced stress responses in bacteria exposed to this herbicide in soils. Induction of SSPs by 2,4-D could be used as a tool in environmental monitoring [3, 22], because their synthesis may serve as a biological indicator by which the presence of toxic environmental pollutants can be established. The synthesis of 2,4-D SSPs can be a sensitive index of stress and the nature of environmental pollution.

In this study, we have examined the degradation of 2,4-D and associated stress protein induction by *Burkholderia* sp. YK-2. The test strain was isolated from contaminated soils that had been treated with 2,4-D. Survival rates of the cells were monitored under 2,4-D stress conditions, and the induction of SSPs was analyzed by SDS-PAGE, 2-D PAGE, and Western blots using anti-DnaK and anti-GroEL monoclonal antibodies.

Materials and Methods

Bacterial isolation and growth conditions. Bacterial enrichment cultures capable of utilizing 2,4-D as the sole source of carbon and

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energy were derived from soil samples that had a previous history of 2,4-D treatment. An isolate that was able to degrade 2,4-D completely at the concentration of 2.25 mM 2,4-D within 36 h was selected for this experiment. The isolate was streaked on trypticase soy agar (TSA) plates for identification, which was based on analysis of fatty acid methyl esters with the Microbial Identification System with a Hewlett-Packard HP 5890 II gas chromatograph. The isolate was maintained in a mineral salts medium that contained up to 2.25 mM 2,4-D [16]. The medium was adjusted to pH 7.0 with 0.5 *N* NaOH before autoclaving. Cultures were grown at 30°C and aerated by shaking on a rotary shaker (New Bruswick Scientific Co., Edison, NJ, USA) at 150 rpm. Growth was monitored by changes in optical density at 660 nm.

2,4-D degradation by the isolate. Degradation of 2,4-D by the isolate was monitored by high performance liquid chromatography (HPLC). The HPLC system consisted of a pump (Shimazu LC-10A, Japan), an injector fitted with a 100-μl loop, UV detector, and integrator. A commercial Zorbax ODS reverse column (250 mm × 4.6 mm, particle size 5 μm) was eluted with a mixture of acetonitrile and phosphate buffer at a flow rate of 1.8 ml min^{-1}. The phosphate buffer contained 6 g of K_2HPO_4 and 3 ml of conc. H_3PO_4 per liter of HPLC-grade water. The detection signal was monitored at 229 nm. Standards of 0.01–2.25 mM 2,4-D used to quantitate residual 2,4-D were prepared from analytical grade 2,4-D purchased from Absolute Standards Inc. (Hamden, CT, USA). Analytical grade 2,4-DCP (2,4-dichlorophenol) was obtained from Absolute Standards Inc., and HPLC-grade acetonitrile and water from Sigma Chemical Co. (St. Louis, MO, USA). The HPLC methodology has been previously described in detail [16].

Stress treatment with 2,4-D and viability test. The cells grown in LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) were harvested by centrifugation at 2000 *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 mineral medium in 100-ml Erlenmeyer flasks containing 0.5–10 mM 2,4-D [22]. The organisms were exposed to 2,4-D in shake flasks at 30°C. After exposure for the proper period, the viable cells were counted by plating them on LB agar.

SDS-PAGE. After the organisms were treated with 2,4-D, the cells were collected by centrifugation at 2000 *g* and suspended in 10 mM phosphate buffer (pH 7.0). Cells in the phosphate buffer were disrupted by ultrasonication (Fisher M-300, Pittsburgh, PA, USA). Prior to SDS-PAGE analysis, the proteins were quantified with a protein assay kit (Sigma Co.) according to the manufacturer's instruction. SDS-PAGE of the proteins was performed according to the method described Bollag et al. [4], with 12% acrylamide for separating gel and 4% acrylamide for stacking gel, respectively, with a running buffer (0.025 M Tris, 0.192 M 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. 2,4-D-treated cells were analyzed for the stress-shock proteins by Western blot technique [19] with anti-DnaK and anti-GroEL monoclonal antibodies (StressGen Biotechnologies Corp., Victoria, BC, Canada), which were induced by heat shocking *Escherichia coli*, known as 70 kDa for DnaK and 60 kDa for GroEL, respectively. The proteins on the gels separated by SDS-PAGE were transferred to Hybond™-PVDF membrane (Amersham International plc., Little Chalfont, Buckinghamshire, England) with a Semiphor semi-dry transfer unit (Owl Separation Systems, Portsmouth, NH, USA). 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 5000 times in PBS–0.08% Tween 20 for 1.5 h. Subsequently, the blots were washed with PBS–0.08% Tween 20. The secondary antibody (anti-mouse IgG HRP conjugate, Promega, Madison, WI, USA) diluted 5000 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.

2-D PAGE. 2-D PAGE was performed according to the methods described by O'Farrell [15] and Bollag et al. [4] with the Bio-Rad Minigel system (Bio-Rad Co., Hercules, CA, USA). The protein samples were mixed with an equal volume of sample buffer (9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1.6% Phamalyte pH 4–6.5, 0.4% Phamalyte pH 3–10) and sample overlay buffer (9 M urea, 0.8% Phamalyte pH 4–6.5, 0.2% Phamalyte pH 3–10, Bromophenol blue). To make 6 ml of isoelectric focusing gel, 3 g of urea was added into a 100-ml side arm flask, and then 0.67 ml of 30% acrylamide and 144 μl of Phamalyte pH 4.0–6.5, 2.7 ml of H_2O were added. The flask was swirled until the urea completely dissolved, and 25 μl of 10% ammonium persulfate and 20 μl N,N,N′,N′-tetramethylethylenediamine (Sigma Co.) were added. The gel was placed onto Bio-Rad Minigel system.

The lower reservoir was filled with 0.01 M H_3PO_4, and the upper reservoir was filled with 0.02 M NaOH. The gel was then pre-run at 200 V for 10 min, 300 V for 15 min, and 400 V for 15 min, consecutively. After the pre-run, the lower and upper buffers were refilled. After the samples were loaded, the gel was run at 500 V for 15 min, and then at 600 V for 3.5 h. After isoelectric focusing, the gel was treated with equilibration buffer (5% β-mercaptoethanol, 62.5 mM Tris-HCl pH 6.8, 2.3% SDS, 10% glycerol) for 30 min. The second dimension was performed according to the SDS-PAGE method described above.

Results and Discussion

Initially, an enriched culture was obtained from soil samples with 2.25 mM 2,4-D as the sole carbon and energy source under aerobic conditions. Six isolates capable of utilizing 2,4-D as the sole carbon source were derived from the original enrichment culture. One isolate was selected on the basis of rapid degradation of the herbicide. The colonies of this isolate were small (<1 mm) in diameter and varied in pigmentation. Microscopic examination of the isolate revealed Gram-negative and rod-shaped cells. The following API-NFT (bioMérieux sa, Marcy-l'Étoile, France) tests were positive: β-galactosidase, arginine dihydrolase, gelatinase, citrate, and sucrose. Negative tests included lysine decarboxylase, ornithine decarboxylase, urease, tryptophanase, acetoin production, glucose, mannitol, inositol, sorbitol, rhamnose, melibiose, amygdalin, arabinose, and oxidase. Fatty acid analyses of this isolate grown on TSA plates were performed to the MIS (Microbial Identification System). On the basis of the results, the isolate could be assigned and designated as *Burkholderia* sp. YK-2.

The degradation of 2,4-D was studied with the *Burkholderia* sp. YK-2 in mineral media under aerobic conditions. When grown with 2,4-D as the sole carbon and

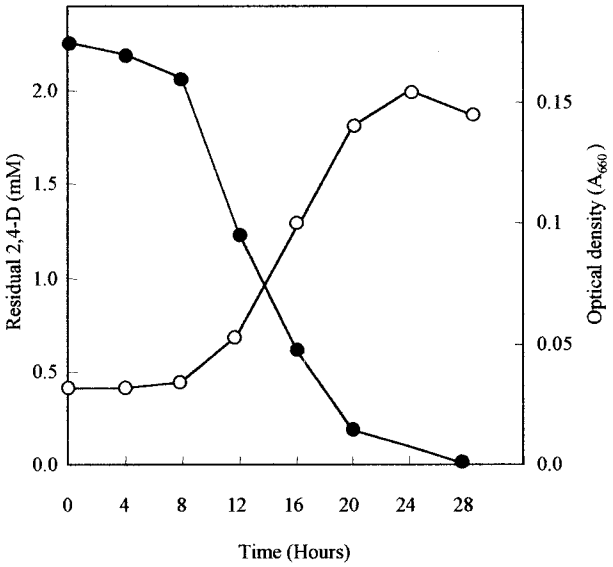

Fig. 1. Growth of *Burkholderia* sp. YK-2 based on optical density at 660 nm (○) and residual 2,4-D concentrations (●).

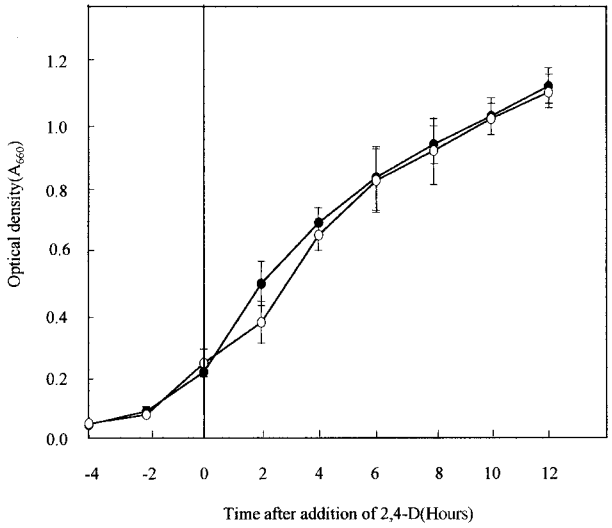

Fig. 2. Growth of *Burkholderia* sp. YK-2 on LB media in the absence and in the presence of 2,4-D. Cells pre-grown overnight on LB medium were harvested, washed in LB medium, and resuspended in LB medium without 2,4-D (●) or with 2 mM 2,4-D (○). 2,4-D was added at time zero, and growth was measured by determination of optical density at 660 nm.

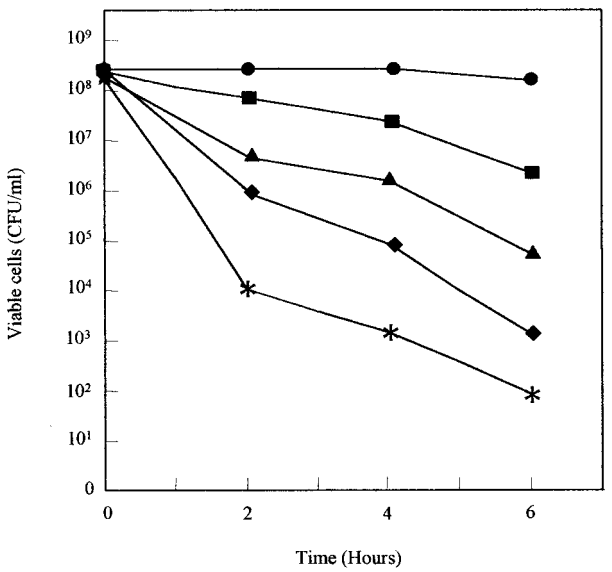

Fig. 3. Survival of *Burkholderia* sp. YK-2 after 2,4-D shock. The cells were maintained at 2,4-D concentrations of 0 mM (●), 1 mM (■), 3 mM (▲), 5 mM (◆) and 7 mM (*), respectively. At intervals, the number of colony-forming units per ml of culture was determined.

energy source, the culture of *Burkholderia* sp. YK-2 completely degraded 2,4-D. Changes in turbidity associated with the biodegradation of 2,4-D are shown in Fig. 1 for the culture of *Burkholderia* sp. YK-2. Growth of these batch cultures typically displayed a lag phase of 8 h before the onset of growth. In general, increases in culture turbidity coincided with parallel decreases in 2,4-D concentration. Excluding the lag phases, the rates of 2,4-D degradation were in the range of approximately 33 mg of 2,4-D liter^{-1} h^{-1} in actively growing batch cultures in shake flasks. Complete depletion of 2,4-D was achieved in this experiment within 28 h of incubation.

Residual 2,4-D in the media was monitored by HPLC. The culture of *Burkholderia* sp. YK-2 produced a metabolite that appeared as an additional peak in the HPLC chromatogram. Further diagnostic work suggested that the metabolite was 2,4-DCP, because an authentic sample of 2,4-DCP displayed an identical retention time to that of the unknown peak. The identification of 2,4-D and 2,4-DCP was confirmed by HPLC analysis of a mixture of analytical grade 2,4-D and 2,4-DCP.

The growth of *Burkholderia* sp. YK-2 was inhibited by the addition of 2,4-D. Fig. 2 illustrates the extent of growth inhibition by 2 mM 2,4-D, which was added to an exponentially growing culture that had reached an optical density at 660 nm of 0.2.

Survival of *Burkholderia* sp. YK-2 was examined during 6 h of incubation in minimal medium containing 0–7 mM 2,4-D, and the results are shown in Fig. 3. The survival rates of cells decreased with increasing (1 mM or higher) concentrations of 2,4-D.

Expression of DnaK and GroEL proteins in *Burkholderia* sp. YK-2 treated with 2,4-D at different concentrations for 10–360 min was evaluated. Induction of DnaK and GroEL proteins was not observed in the cells treated with 2,4-D for 10 min at 0.5–10 mM concentrations. The DnaK protein was not produced in the cells

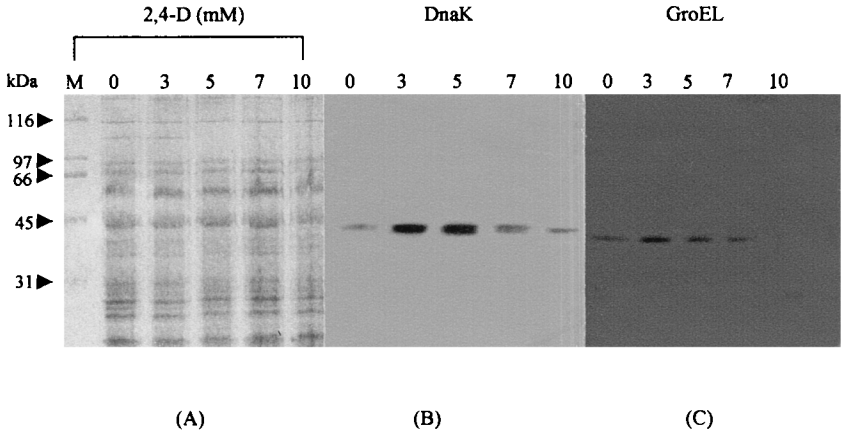

Fig. 4. Induction of SSPs by *Burkholderia* sp. YK-2 treated with different 2,4-D concentrations for 1 h. The SSPs were analyzed by SDS-PAGE (A) and by Western blot with anti-DnaK (B) and anti-GroEL (C) monoclonal antibodies, respectively.

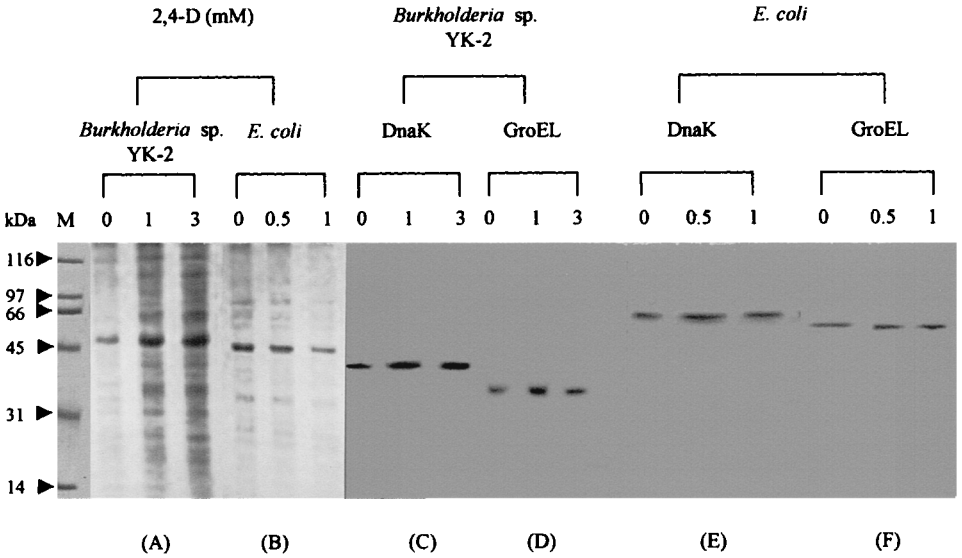

Fig. 5. Induction of SSPs by *Burkholderia* sp. YK-2 and *E. coli* treated with different 2,4-D concentrations for 1 h. The SSPs were analyzed by SDS-PAGE (A and B), and Western blot with anti-DnaK and anti-GroEL monoclonal antibodies in *Burkholderia* sp. YK-2 (C and D) and *E. coli* (E and F), respectively.

treated with 2,4-D for 30 min. Both DnaK and GroEL proteins began to appear in the cells treated with 7 mM 2,4-D for 30–60 min. However, no GroEL protein was induced under these conditions with 10 mM 2,4-D concentration in this period. The proteins were detected in the cells that were treated with between 0.5 and 5 mM 2,4-D for 180 min or longer.

The DnaK and GroEL stress shock proteins 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 stress shock proteins produced with 2,4-D treatment are shown in Figs. 4B and 4C, respectively.

2,4-D stress shock response was characterized by comparing the molecular mass of DnaK and GroEL proteins induced in *Burkholderia* sp. YK-2 and *E. coli*. Results from this study demonstrated that *E. coli* responded to this stress by the production of these proteins, two of which were the 70-kDa DnaK protein and the 60-kDa GroEL protein (Fig. 5).

However, the molecular masses of induced DnaK and GroEL proteins in *Burkholderia* sp. YK-2 were approximately 43kDa and 41 kDa, respectively, and each size was identical to the molecular mass of the proteins treated with heat shock, shifted from 30°C to 42°C for 30 min (Fig. 6).

2-D PAGE was used for the separation and analysis of SSPs from *Burkholderia* sp. YK-2 (Fig. 7). Fig. 7B

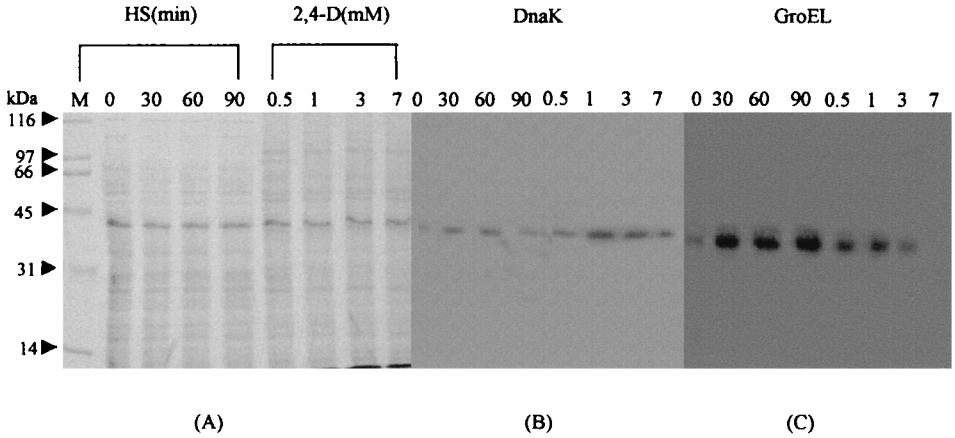

Fig. 6. Induction of SSPs by *Burkholderia* sp. YK-2 treated with heat shock (HS) at 42°C and 2,4-D (A) as a stress shock. The SSPs were analyzed by SDS-PAGE (A), and Western blot with anti-DnaK (B) and anti-GroEL (C) monoclonal antibodies, respectively.

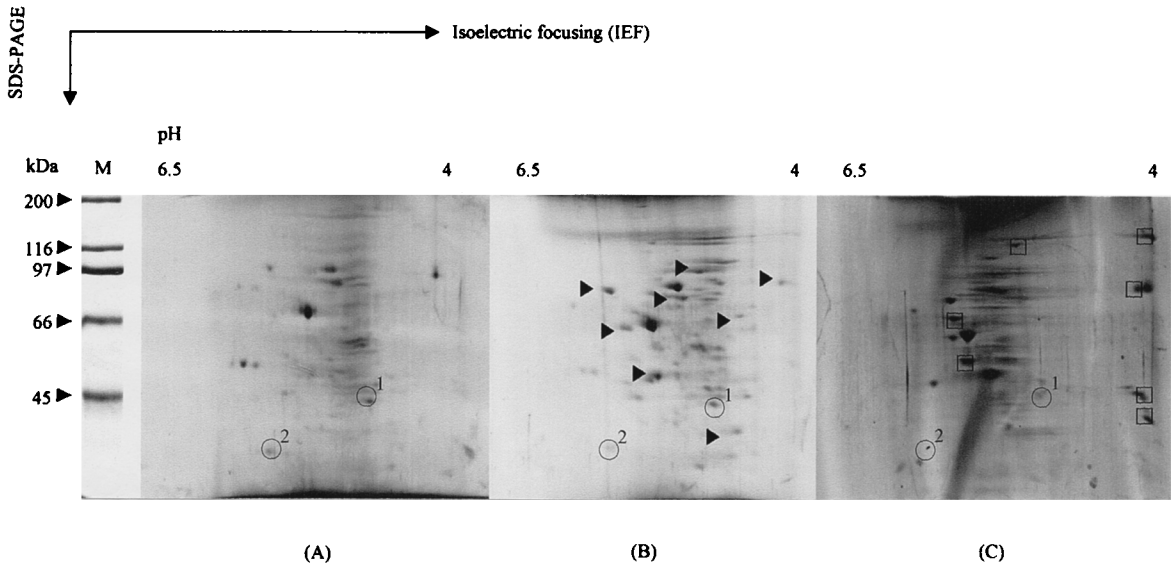

Fig. 7. 2-D PAGE analysis of SSPs of *Burkholderia* sp. YK-2; control cells (A), cells treated with 3 mM 2,4-D for 1 h (B), and cells treated with 7 mM 2,4-D for 1 h (C). The circle spots marked with DnaK (1) and GroEL (2) proteins. Arrow heads and open rectangles indicate the proteins positively induced by 2,4-D shock.

and 7C show the induction of several remarkable proteins including DnaK and GroEL in response to 2,4-D treatment for 1 h. This analysis shows that the induction of 2,4-D SSPs depends on the concentration of 2,4-D in the medium.

Thus, in this study we demonstrated the synthesis and requirement of DnaK and GroEL for survival under 2,4-D stress in *Burkholderia* sp. YK-2. The cells treated with 3 mM and 7 mM 2,4-D produced about 15 SSPs. In general, DnaK is a member of the Hsp 70 family, and GroEL of the Hsp 60 family [5]. Varela et al. [23] reported that the major heat shock proteins from another proteobacterium, *Thiobacillus ferrooxidans* were identified as DnaK and GroEL equivalents by Western blot and analysis of the N-terminal amino acid sequence of spots isolated from 2-D PAGE. *Thiobacillus ferrooxidans* chaperonins showed 70% and 80% identity with the *E. coli* GroEL and DnaK, respectively. The GroEL proteins from both bacteria formed a 14-mer, whereas *E. coli* DnaK protein existed partially as a dimer [23].

Several studies have demonstrated that degradation and survival rate of organisms diminish at high concentration of aromatic hydrocarbons [12, 14, 24]. Toxic effects were recognized to be due to the permeability change in membrane, attributed to fatty acids [9] and energy depletion caused by change of glycolytic metab-

olite levels in the benzoate degradation pathway [25]. These findings suggest that SSPs such as DnaK and GroEL are required for the cells to survive under 2,4-D stress. This is the first report, to our knowledge, of a situation where 2,4-D serves both as a substrate and as an inducer of a stress response in a soil bacterium. Whether other pesticides elicit similar stress responses in degrader microorganisms is not known. Future work will investigate expression and functions of stress proteins in this soil bacterium under various environmental pollutant stresses.

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