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TOXIC EFFECTS OF 2,4-D AND OTHER AROMATIC COMPOUNDS ON BACTERIA, AND THEIR PROTECTIVE RESPONSES

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The purpose of this work was to investigate the induction of stress shock proteins (SSPs) in *Burkholderia* sp. YK-2 in response to 2,4-dichlorophenoxyacetic acid (2,4-D), and *Pseudomonas* sp. DJ-12 to benzoate, 4-chlorobenzoate (4-CBA), 4-hydroxybenzoate, and biphenyl. The SSPs, which contribute to the resistance of the cytotoxic effect of the toxic aromatic compounds including 2,4-D and 4-CBA, were induced at different concentrations of the compounds in exponentially growing cultures of *Burkholderia* sp. YK-2 or *Pseudomonas* sp. DJ-12. This response involved the induction of a 43 kDa DnaK and 41 kDa GroEL proteins in *Burkholderia* sp. YK-2, characterized by SDS-PAGE and Western blot using the anti-DnaK and anti-GroEL monoclonal antibodies. In *Pseudomonas* sp. DJ-12, 70 kDa DnaK and 60 kDa GroEL proteins was induced as SSPs, respectively. The total SSPs were analyzed by 2-D PAGE. Survival of *Burkholderia* sp. YK-2 or *Pseudomonas* sp. DJ-12 with time in the presence of different concentrations of the compounds was monitored, and viable counts paralleled the induction of the SSPs in these strains. Cells treated with the increased concentrations of toxic compounds showed some destructive openings on the cell envelopes.

INTRODUCTION

Exposure of indigenous microoganisms 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, trichloroethylene were reported to induce SSPs [2, 3, 6, 25]. Chemicals have been shown to stimulate the synthesis of SSPs in *Escherichia coli* [3, 6, 12] and *Pseudomonas putida* [9, 15, 18, 19, 27], and some of these responses are chemical-specific. Thus, the precise cellular response to a specific stress is characteristic to the chemical. The lipophilic properties of toxic aromatic hydrocarbons are known to affect the cytoplasmic membrane, resulting in the destruction of the membrane, changes in permeability, loss of magnesium and calcium ions, and denaturation of membrane proteins [22, 23]. Aromatic

compounds such as benzoate destroy the pH gradient and denature some enzyme proteins in cells. Toluene and benzoate have been reported to change cell shape and the calcium homeostasis in the cells [13].

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 [24]. 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, 25], 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 toxic aromatic compounds, and associated stress protein induction by *Burkholderia* sp. YK-2 and *Pseudomonas* sp. DJ-12. The test strains were isolated from contaminated soils that had been treated with 2,4-D or toxic aromatic compounds. Survival rates of the cells were monitored under the stress conditions of the compounds and the induction of SSPs was analyzed by SDS-PAGE, 2-D-PAGE, and Western blots using anti-DnaK and anti-GroEL monoclonal antibodies. In addition, the survival resistance was studied in correlation of the production of SSPs and morphological changes induced by the stresses of sublethal conditions.

MATERIALS AND METHODS

Bacterial isolation and growth conditions

Bacterial enrichment cultures capable of utilizing 2,4-D as the sole source of carbon and 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 using 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. Similar experimental procedure was followed for isolation, identification and maintenance of *Pseudomonas* sp. DJ-12.

Degradation of toxic compound by the isolate

Degradation of 2,4-D by the isolate was monitored using high performance liquid chromatography (HPLC). The HPLC system was consisted of a pump (Shimazu LC-10A, Japan), an injector fitted with 100 μl loop, UV detector, and integrator. A commercial Zorbax ODS reverse column (250 mm x 4.6 mm, particle size 5 μ m) was eluted with a mixture of acetonitrile and phosphate buffer at a flow rate of 1.8 ml/min. 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 [17].

Stress treatment with toxic compound and viability test

The cells grown in LB broth were harvested by centrifugation at 2,000 x *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 in 30 ml mineral medium in 100 ml Erlenmeyer flasks containing 0.5 to 10 mM 2,4-D [22]. The organisms were exposed to 2,4-D in shake flasks at 30°C. After exposure for proper period, the viable cells were enumerated by plating them on LB agar.

SDS-PAGE

After the organisms were treated with 2,4-D, the cells were collected by centrifugation at 2,000 x *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., St. Louis, MO, USA) according to the manufacturer's instruction. SDS-PAGE of the proteins was performed according to the method described Bollag *et al.* [4] using 12% acrylamide for separating gel and 4% acrylamide for stacking gel, respectively, with a running buffer at 60-90 V for 2.5 h. Gels were stained with a staining solution 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 SSPs by Western blot technique [20] using 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^{TM}-PVDF membrane (Amersham International plc., Buckinghamshire, England) with a Semiphor semi-dry transfer unit (Owl Separation Systems, Porthmouth, 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 5,000 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 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.

2-D PAGE

2-D PAGE was performed according to the methods described by O'Farrell [16] and Bollag *et al.* [4] using the Bio-Rad Minigel system (Bio-Rad Co., Hercules, CA, USA). The protein samples were mixed with 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 TEMED 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 minutes, 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.

Scanning Electron Microscopy

The cells of *Burkholderia* sp. YK-2 and *Pseudomonas* sp. YK-2 grown in MM2 were treated with 2,4-D or 4-CBA for each 1 h. The cells collected on 0.2 μm membrane filter (Millipore Co., Bedford, MA, USA) by gentle filtration were pre-fixed with 2.5% glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.2) for 2 h, and then post-fixed cells with 1% osmium tetroxide in the same buffer. The fixed cells were dehydrated with a serial concentration (30 to 95%) of ethanol for every 15 min, and then 100% ethanol for 20 min. The cells were substituted with absolute isoamyl acetate for 15 min, and then air-dried. The cells were coated with gold using a sputter coater (IB-3, Giko Engineering Co., Tokyo, Japan) and examined with a scanning electron microscope (S-2500C, Hitachi Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Initially, enriched cultures were obtained from soil samples with 2,4-D or 4-CBA

as the sole carbon and energy source under aerobic conditions. Several isolates capable of utilizing 2,4-D or 4-CBA as the sole carbon source were derived from the original enrichment culture, respectively. Single isolate was selected on the basis of rapid degradation of each compounds, respectively. Microscopic examination of each isolate revealed Gram-negative and a rod-shaped cell. Biochemical tests were performed to characterize the isolates using API-NFT (bioMérieux sa, Marcy-lÉtoile, France). Fatty acid analyses of this isolate grown on TSA plates were performed to the MIS (Microbial Identification System). Based on the results, the isolates could be assigned and designated as *Burkholderia* sp. YK-2 and *Pseudomonas* sp. DJ-12, respectively.

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 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 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/h in actively growing batch cultures in shake flasks. Complete depletion of 2,4-D was achieved in this experiment within 28 h of incubation. Similar experimental procedure was performed to *Pseudomonas* sp. DJ-12.

Burkholderia sp. YK-2 and *Pseudomonas* sp. DJ-12 cells were treated with various concentrations of 2,4-D or 4-CBA to examine the cell viability under the toxic aromatic compound stress. The cell viability rates of *Burkholderia* sp. YK-2 cells decreased with increasing (1 mM or higher) concentrations of 2,4-D. *Pseudomonas* sp. DJ-12 grew on 4-CBA at concentrations of 0.5 and 1.0 mM even after 30 h of incubation. However, the cell viability at a 3 mM or higher concentrations of 4-CBA decreased the survival by acting as stress to the cells.

The morphological changes of *Burkholderia* sp. YK-2 and *Pseudomonas* sp. DJ-12 cells were examined by exposure to lethal conditions of each stress which caused a significant decrease in their ability. As seen in the scanning electron micrographs, normal cells exhibited a typical rod shape with smooth surfaces. However, the cells treated with 2,4-D or 4-CBA showed some destructive openings on the cell envelopes.

Expression of DnaK and GroEL proteins in *Burkholderia* sp. YK-2 treated with 2,4-D

and *Pseudomonas* sp. DJ-12 with 4-CBA 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 to 10 mM concentrations. The DnaK protein was not produced in the cells 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 to 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 between 0.5 to 5 mM 2,4-D for 180 min or longer. 4-CBA induced DnaK at concentrations of 0.5 mM or higher, and GroEL proteins were also produced by treatment with 4-CBA with the same pattern as DnaK production.

The DnaK and GroEL SSPs were detected by SDS-PAGE and Western blot with anti-DnaK and anti-GroEL monoclonal antibodies. Both profiles of total proteins and DnaK and GroEL SSPs produced with 2,4-D treatment are shown in Figs., 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 protains, two of which were the 70 kDa DnaK protein and the 60 kDa GroEL protein. However, the molecular mass 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. In *Pseudomonas* sp. DJ-12, 70 kDa DnaK and 60 kDa GroEL proteins were induced as SSPs, and revealed that each size of these SSPs was identical to the molecular mass of the proteins induced by *E. coli.*

2-D PAGE was used for the separation and analysis of SSPs from *Burkholderia* sp. YK-2 and *Pseudomonas* sp. DJ-12. Induction of several remarkable proteins including DnaK and GroEL in response to 2,4-D or 4-CBA treatment for 1 h was observed. This analysis shows that the induction of 2,4-D or 4-CBA SSPs depends on the concentration of 2,4-D or 4-CBA in the medium.

Several studies have demonstrated that degradation and survival rate of organisms diminish at high concentration of aromatic hydrocarbons [12, 14, 27]. 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 metabolite 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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