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Responses of *Pseudomonas* sp. DJ-12 to Pollutant Stresses of Benzoate and 4-Chlorobenzoate

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Abstract Aromatic hydrocarbons can be utilized as carbon and energy sources by some microorganisms at lower concentrations. However, they can also act as stresses to these organisms at higher concentrations. *Pseudomonas* sp. DJ-12 is capable of degrading 0.5 mM concentration of benzoate and 4-chlorobenzoate (4CBA). In this study, the exposure of *Pseudomonas* sp. DJ-12 to the pollutant stresses of benzoate and 4CBA at various concentrations was comparatively studied for its cellular responses, including survival tolerance, degradability of the aromatics, and morphological changes. *Pseudomonas* sp. DJ-12 utilized 0.5 to 1.0 mM benzoate and 4CBA as carbon and energy sources for growth. However, the organism could not degrade the aromatics at concentrations of 3 mM or higher, resulting in reduced cell viability due to the destruction of the cell envelopes. *Pseudomonas* sp. DJ-12 cells produced stress-shock proteins such as DnaK and GroEL when treated with benzoate and 4CBA at concentrations of 0.5 mM, or higher as sublethal dosage; Yet, there were differing responses between the cells treated with either benzoate or 4CBA. 4CBA affected the degradability of the cells more critically than benzoate. The DnaK and GroEL stress-shock proteins were produced either by 1 mM benzoate with 5 min treatment or by higher concentrations after 10 min. The proteins were also induced by 0.5 mM 4CBA, however, it needed at least 20 min treatment or longer. These results indicate that the chlorination of benzoate increased the recalcitrance of the pollutant aromatics and changed the conditions to lower concentrations and longer treatment times for the production of stress-shock proteins. The stress-shock proteins produced by the aromatics at sublethal concentrations functioned interactively between the aromatics for survival tolerance to lethal concentrations.

Key words: Stress response, benzoate, 4-chlorobenzoate, stress-shock proteins, *Pseudomonas* sp. DJ-12

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When living organisms including prokaryotic bacteria are exposed to environmental stresses, they are capable of adapting to a new environment by changing and regulating the expression of their genetic characteristics. Such adaptability to extreme environments has evolved differently even among bacterial species. The responses of bacteria to particular environmental stresses including heat shock [21], oxidative stress [2, 9], anaerobiosis [31], and others [10, 11, 12, 14] have been extensively studied.

Recently, pollutant chemicals contaminating the natural ecosystem have been included as environmental stresses that disturb the endocrine hormone systems of higher organisms. The major group of pollutant chemicals is recognized to be aromatic hydrocarbons, including halogenated and polycyclic aromatics, such as polychlorinated biphenyl and dioxin compounds [3]. The lipophilic properties of pollutant 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 [29, 30]. Sikkema *et al.* [30] and Warth [32] reported that aromatic hydrocarbons 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 [22].

In order for cells to protect themselves from the influences of pollutant stresses, they produce various kinds of particular proteins which are called stress-shock proteins (SSPs). In general, such stress-shock proteins, including DnaK and GroEL, are recognized to involve in the maturation of newly synthesized proteins and in the refolding or degradation of denatured proteins [20]. Accordingly, it is believed that their importance during environmental stresses is in rescuing or scavenging stress-denatured proteins [15, 17].

Faber *et al.* [10] reported that several kinds of SSPs were newly synthesized by the induction of stress-shock genes in *E. coli* when treated with 10 mg/l pentachlorophenol or 200 mg/l monochlorophenol. The production of stress-

shock proteins was also reported in *Pseudomonas putida* KT2442 treated with 2-chlorophenol [23]. Other aromatic hydrocarbons, such as benzene, 2,4-dichloroaniline, dioctylphtalate, and tetrapropylbenzosulfonate, have also been recognized to produce stress-shock proteins, about 50% of which are identical to those induced by heat shock and carbon starvation [4].

The production of stress-shock proteins has also been studied in *Pseudomonas* sp. DJ-12 treated with various pollutant aromatics [26, 27]. *Pseudomonas* sp. DJ-12 has also been reported to degrade biphenyl and benzoate at lower concentrations [18, 19]. Accordingly, in this study, *Pseudomonas* sp. DJ-12 cells treated with higher concentrations of benzoate were studied for their cellular responses including survival, tolerance, degradability of benzoate, and morphological cell changes. 4-Chlorobenzoate was also compared with benzoate for the same cellular responses.

MATERIALS AND METHODS

Growth of the Strain on Benzoate Compounds

The bacterial strain used in this study was *Pseudomonas* sp. DJ 12 which was isolated from wastewater as a biphenyl and benzoate degrader [18]. The strain is capable of degrading 4-chlorobenzoate (4CBA) as well as benzoate at a 0.5 mM concentration [6]. The cells of *Pseudomonas* sp. DJ 12 collected from a Luria Bertani (LB) broth culture were treated with benzoate or 4CBA added to a MM2 medium at various concentrations [26]. The cells were sampled at appropriate times during a 30 min treatment and were examined for their viability by plating on LB agar at 30°C. Their degradability of benzoate and 4CBA was evaluated by the resting cell assay as described by Arensdorf and Focht [1].

Scanning Electron Microscopy

Colonies of *Pseudomonas* sp. DJ-12 grown on LB agar plates for 12 h were extracted as blocks of 0.5 cm^3. Those agar blocks with a colony were then exposed to a 7 mM concentration of benzoate or 4CBA in a MM2 medium for one hour. The colonies treated with the compounds were pre-fixed with 2.5% glutaraldehyde in a 100 mM potassium phosphate buffer (pH 7.2) for 2 h, and then post-fixed with 1% osmium tetraoxide in the same buffer as described by Ng *et al.* [25]. 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., Japan) and then examined under a scanning electron microscope (S-2500C, Hitachi Co., Japan).

Detection of Stress-Shock Proteins

Pseudomonas sp. DJ-12 cells (10^7- 10^8 CFU/ml) were treated with various concentrations of benzoate or 4CBA in a MM2 medium. The cells were taken out of the suspension at appropriate times during a 60 min treatment, pelleted by centrifugation at 3,000 ×g for 5 min, and then washed twice with a 10 mM phosphate buffer. The cells were resuspended in a 10 mM phosphate buffer (pH 7.0) and were sonicated (by Labsonic 2000, Laboratory Supply Co., Germany) for 15 sec 15 to 20 times repeatedly on ice.

The purified proteins were analyzed by SDS-PAGE according to the method of Bollag *et al.* [5] using 10% and 4% acrylamide for separation and stacking gels, respectively. The proteins were quantified with a protein assay kit (Sigma Co., U.S.A.) according to the manufacturer's instruction. Electrophoresis, staining with Coomassie Brilliant blue, and destaining of the gels were followed by the procedures described by Park *et al.* [27].

The DnaK and GroEL stress-shock proteins were analyzed by a western blot using anti-DnaK and anti-GroEL monoclonal antibodies (StressGen Biotechnologies Corp., Canada) as described by Sambrook *et al.* [28]. The proteins on the gels separated by SDS-PAGE were transferred to a Hybond™-PVDF membrane (Amersham International plc., England) with a Semiphor semi-dry transfer unit (Hoefer Scientific Instruments, U.S.A.). The reaction of the proteins with the antibodies and detection of the immunocomplexes were processed according to the manufacturer's instructions.

Survivorship Test

In order to induce the stress-shock proteins in *Pseudomonas* sp. DJ-12, about 10^7- 10^8 cells/ml were treated with either 1 mM benzoate in a MM2 medium for 10 min or 4CBA in the same medium for 20 min. The pre-shocked cells were harvested by centrifugation at 2,000 ×g and then challenged to higher concentrations (7 mM) of benzoate or 4CBA in MM2 as described by Park *et al.* [26]. The survival of the cells was examined at appropriate intervals during 60 min of incubation by enumerating the colonies developed on the LB plate at 30°C.

RESULTS AND DISCUSSION

Degradation of Benzoate and 4CBA

Benzoate and 4-chlorobenzoate (4CBA) at concentrations of 0.5 and 1.0 mM were degraded by *Pseudomonas* sp. DJ-12 as shown in Table 1. Both aromatic compounds, however, were not degraded by the organism at concentrations of 3 mM or higher. The benzoates at 0.5 to 1.0 mM began to be degraded 6 h after incubation, however, degradation of 4-chlorobenzoate at the same concentrations was

delayed until after 19 h of incubation. This indicated that the chlorination of aromatic hydrocarbons increases the recalcitrance of the compounds, as indicated by Furukawa *et al.* [13] and Chaudry and Chapalamadugu [7], on chlorinated aromatic hydrocarbons such as 4- chlorobiphenyl, chlorophenol, and chlorobenzoate.

Table 1. Degradation of benzoate and 4-chlorobenzoate by *Pseudomonas* sp. DJ-12.

Aromatic compounds	Incubation time (h)	Degradation at different concentrations (mM)				
		0.5	1	3	5	7
BA	1	-	-	-	-	-
	6	++	+	-	-	-
	19	+++	+++	-	-	-
	30	+++	+++	-	-	-
4-CBA	1	-	-	-	-	-
	6	-	-	-	-	-
	19	+++	++	-	-	-
	30	+++	+++	-	-	-

+++, 80% degradation; ++, 60% degradation; +, 40% degradation; -, no degradation.
Abbreviations: BA, benzoate; 4CBA, 4-chlorobenzoate.

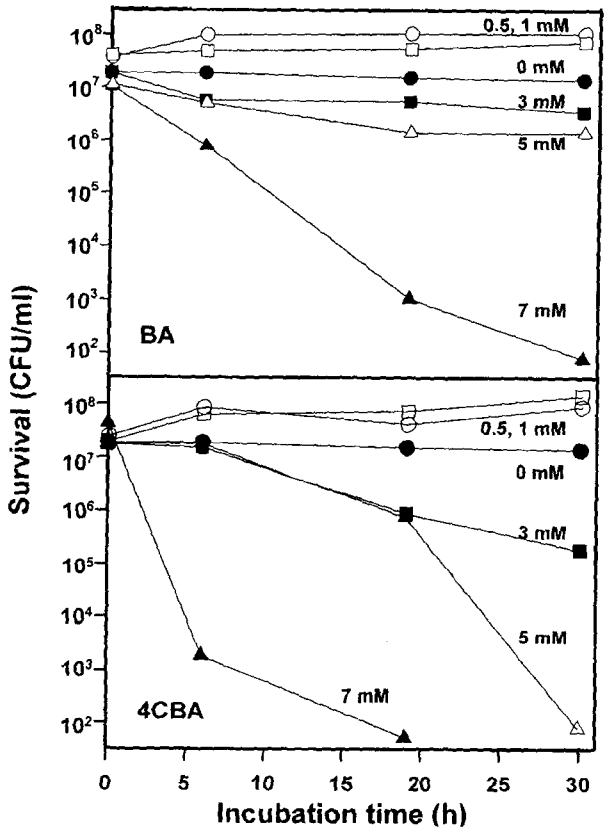

Fig. 1. Viability of *Pseudomonas* sp. DJ-12 treated with benzoate or 4-chlorobenzoate at various concentrations.

Cell Viability under Pollutant Stresses

Pseudomonas sp. DJ-12 cells (10^7- 10^8 CFU/ml) were treated with various concentrations of benzoate or 4CBA in order to examine the cell viability under chemical stress. The results are shown in Fig. 1. *Pseudomonas* sp. DJ-12 grew on benzoate and 4CBA at concentrations of 0.5 and 1.0 mM even after 30 h of incubation. However, culturable cell numbers of the organisms decreased by benzoate compounds at concentrations of 3 mM or higher.

This indicates that 0.5 to 1.0 mM concentrations of the compounds were utilized as carbon and energy sources by this organism, however, a 3 mM or higher concentration decreased cell viability by acting as a stress to the cells. These results were in good agreement with degradation of

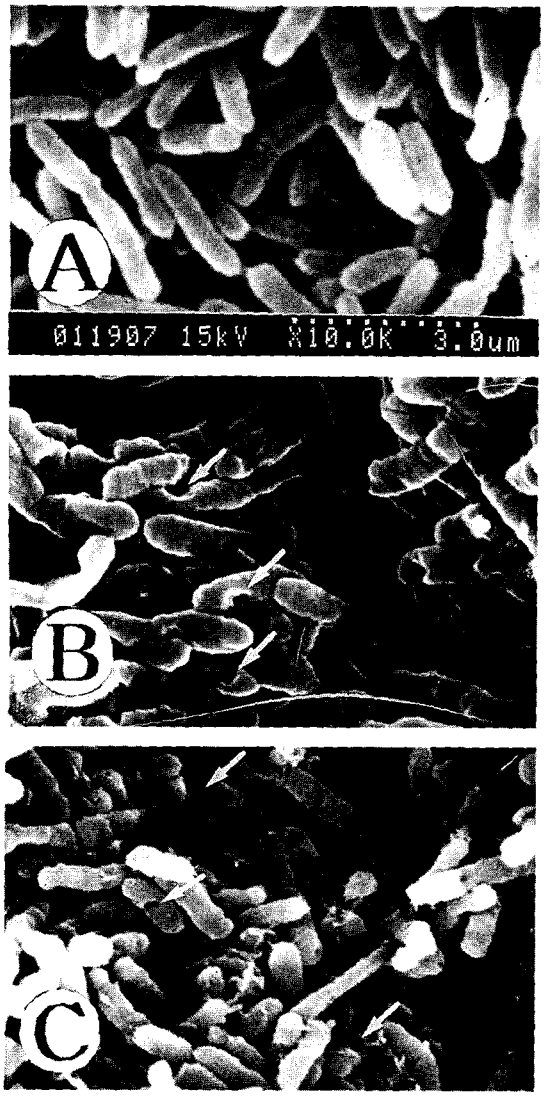

Fig. 2. Scanning electron micrographs of *Pseudomonas* sp. DJ-12 treated with benzoate or 4-chlorobenzoate.
A, untreated cells; B, cells treated with 7 mM benzoate for 30 min; C, cells treated with 7 mM 4CBA for 30 min. Arrows indicate the destroyed cell envelopes.

the organism with benzoate compounds at the same concentrations, as shown in Table 1. Blasco *et al.* [3] reported that 4-chlorobenzoate, 4-chlorocatechol, and 4-chlorobiphenyl all inhibited the growth of *Pseudomonas* sp. at higher concentrations which could not be degraded by the organism.

Effect of Benzoate Compounds on Morphology

In order to examine the effects of the chemicals on cellular morphology, *Pseudomonas* sp. DJ-12 cells were exposed to 7 mM concentrations of benzoate or 4CBA which caused a significant decrease in viability. The scanning electron micrographs of the cells are shown in Fig. 2. Normal cells exhibit a typical rod shape with smooth surfaces (Fig. 2A), however, the cells treated with 7 mM benzoate for 30 min (Fig. 2B) showed some destructive openings (arrows) on the cell envelopes and irregular rod forms with wrinkled surfaces. Those cells treated with 7 mM 4CBA (Fig. 2C) also indicated some destruction (arrows) of the cell walls and irregular morphology of the organism. Such morphological changes of the cells by aromatic hydrocarbons had been reported previously by Crude *et al.* [8] and Liu and Fechter [22]. The cell membranes of *Pseudomonas putida* were equally destroyed by exposure to toluene and xylene [5].

Production of Stress-Shock Proteins

The *Pseudomonas* sp. DJ-12 cells which were treated with various concentrations of either benzoate for 10 min or 4CBA for 30 min exhibited the production of DnaK stress-shock proteins as shown in Fig. 3. Benzoate induced the production of DnaK at concentrations of 1 mM or higher, and 4CBA induced DnaK at concentrations of 0.5 mM or higher. GroEL proteins were also produced by treatment with benzoate or 4CBA with the same pattern as DnaK production (Fig. 4). However, the amount of GroEL induced by 4CBA was slightly less than that by benzoate.

The production of DnaK and GroEL stress-shock proteins by various concentrations of benzoate and 4CBA is summarized in Table 2. Benzoate induced the production

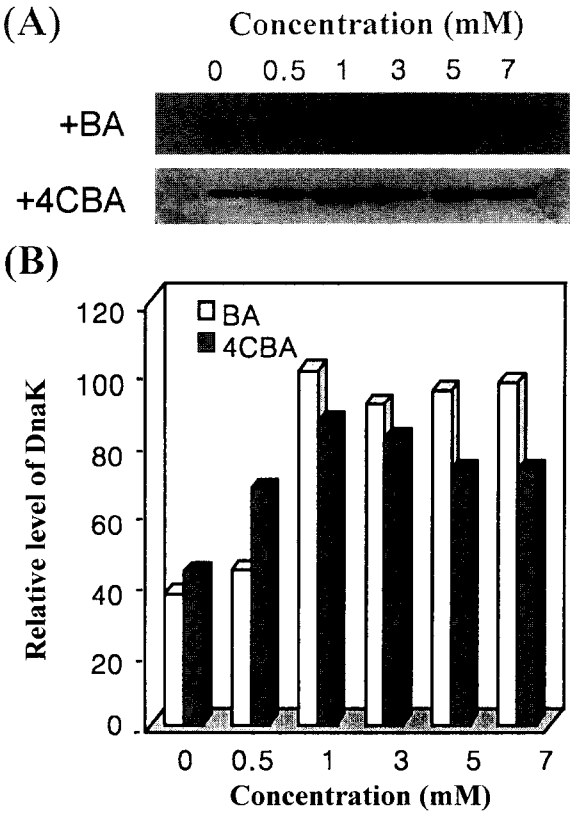

Fig. 3. Production of DnaK by *Pseudomonas* sp. DJ-12 treated with benzoate or 4-chlorobenzoate.
A, Western blot of DnaK produced in the cells treated with benzoate (BA) at various concentrations for 10 min or 4-chlorobenzoate (4CBA) for 30 min; B, densitometric analysis of the blot.

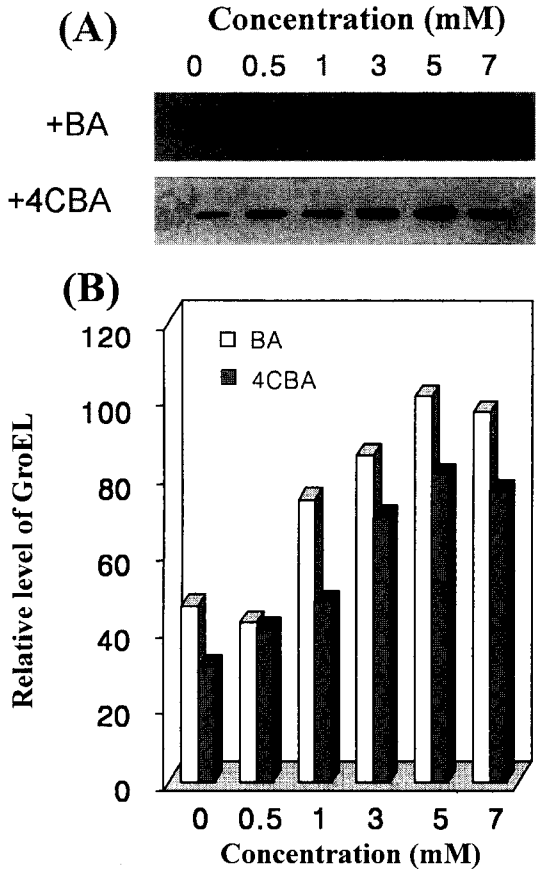

Fig. 4. Production of GroEL by *Pseudomonas* sp. DJ-12 treated with benzoate or 4-chlorobenzoate.
A, Western blot of GroEL produced in the cells treated with benzoate (BA) at various concentrations for 10 min or 4-chlorobenzoate (4CBA) for 30 min; B, densitometric analysis of the blot.

Table 2. Production of stress-shock proteins (SSP) by *Pseudomonas* sp. DJ-12 treated with benzoate and 4-chlorobenzoate at different concentrations.

Treatment time (min)	Stress-shock protein	SSP production at concentrations of benzoate (mM)					SSP production at concentrations of 4-chlorobenzoate (mM)				
		0.5	1	3	5	7	0.5	1	3	5	7
5	DnaK	-	+	+	+	+	-	-	-	-	-
	GroEL	-	-	-	-	-	-	-	-	-	-
10	DnaK	-	+	+	+	+	-	-	-	-	-
	GroEL	-	+	+	+	+	-	-	-	-	-
20	DnaK	-	+	+	+	+	±	±	±	±	±
	GroEL	-	+	+	+	+	+	+	+	+	+
30	DnaK	-	+	+	+	+	+	+	+	+	+
	GroEL	+	+	+	+	+	±	±	+	+	+
60	DnaK	-	-	+	+	+	+	+	+	+	+
	GroEL	-	-	-	-	-	-	±	±	±	±

-, no production; ±, weak production; +, heavy production.

of the proteins after 5 min of treatment or longer at any concentration. However, the induction of the stress-shock proteins by 4CBA took at least 20 min or longer. Benzoate induced DnaK after 5 min of treatment, yet it took 10 min to produce GroEL. Conversely, 4CBA could induce the production of both DnaK and GroEL after 20 min of treatment or longer. Faber *et al.* [10] reported that *E. coli* K12 produced 23 kinds of stress-shock proteins in response to chlorophenol stress. Nine different pollutant chemicals were reported to induce the production of 13 to 39 kinds of stress-shock proteins in *E. coli*, some of which were identical to those produced by heat shock and carbon starvation [4]. The reduction of the stress-shock proteins in the cells treated with 4CBA at concentration of 5 mM or higher could be explained by the reduction of sigma-32 by longer treatment with 4CBA at higher concentrations. The sigma-32 is recognized to be needed for synthesis of the proteins as indicated by Mager and De Kruijff [24]. Kanemori *et al.* [16] reported that the stress-shock proteins and sigma-32 were concomitantly reduced in the cells treated with chemicals at higher concentrations than those for maximum production of the proteins.

Survivorship under the Lethal Concentrations of Pollutants

The cells pre-shocked with 1 mM benzoate or 4CBA exhibited a certain degree of survival tolerance to higher concentrations (7 mM) of the compounds as shown in Fig. 5. When the pre-shocked cells (10^6- 10^7 CFU/ml) were exposed to 7 mM benzoate or 4CBA for 60 min, the numbers of surviving cells were only slightly reduced compared to a decrease of 2 to 3 orders of magnitude in the untreated control cells.

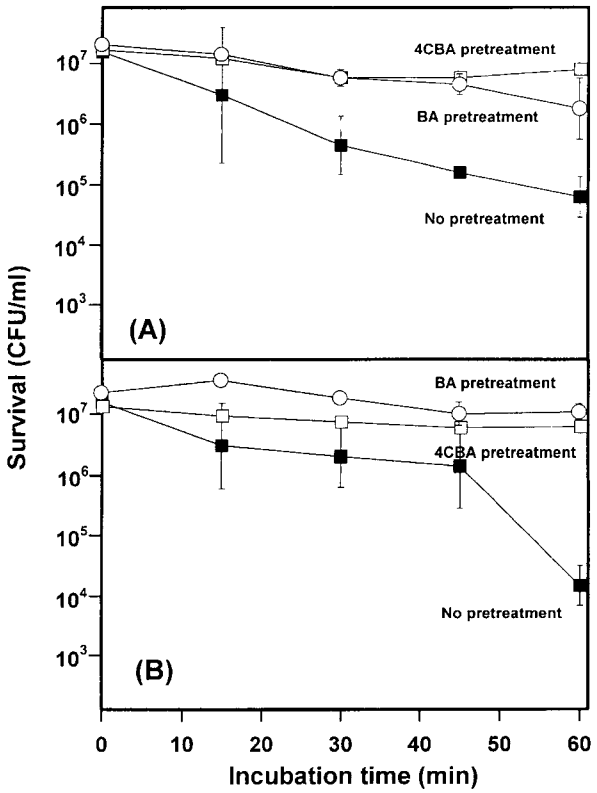

Fig. 5. Survival tolerance to 7 mM benzoate (A) and 4-chlorobenzoate (B) in *Pseudomonas* sp.
DJ-12 cells pretreated with 1 mM benzoate (BA) for 10 min (○) or 1 mM 4-chlorobenzoate (4CBA) for 20 min (□), and untreated cells (■).

Various organisms are capable of adapting to lethal conditions when pretreated with a sublethal condition for a short time [11, 12]. Blom *et al.* [4] reported that *E. coli* cells treated with pollutant chemicals at a concentration that did not inhibit their growth became tolerant to higher concentrations through the production of stress-shock proteins. DnaK and GroEL proteins were also produced in the cells which were pre-shocked with 1 mM benzoate or 4CBA as seen in Table 2. Such concentrations of the compounds were, therefore, utilized as carbon and energy sources for the cells to grow, as shown in Table 1. Accordingly, *Pseudomonas* sp. DJ-12 cells pretreated with 1 mM benzoate or 4CBA proved to be tolerant to higher concentrations of the compounds. Such a tolerance was found to function similarly between both 4CBA and benzoate.

In summary, *Pseudomonas* sp. DJ-12 could utilize benzoate and 4CBA for growth at 1 mM concentration or lower. The DnaK and GroEL stress-shock proteins were synthesized by the aromatics at sublethal concentrations, and the proteins functioned interactively between both aromatics for survival tolerance to lethal concentrations.

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