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Degradative and Protective Functions of Microorganisms to Toxic Aromatic Pollutants

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I. Aromatic pollutants as environmental stress

What is stress for living things including microorganisms? It is difficult to provide an universal definition for stress, since the perception of a stressful situation is highly dependent on the individual cell. Nevertheless, stress can be any deviation from optimal growth conditions that results in a reduced growth rate. Stress also can be defined as exposure to any environmental situation that results in damage of cellular components in the absence of a cellular response. Such definition needs something more about the physiology of the response. Furthermore, stress may be defined as a situation that stimulates the expression of genes known to respond to a specific environmental condition.

There are different levels of stress severity: ① under minor stress, growth generally continues at the same rate and cells fully adapt to the new conditions. ② under severe stress, the growth rate is reduced but cells can still adapt and tolerate the situation, albeit at a high price. ③ under extreme stress, growth ceases and cells devote their resources to survival. ④ under lethal stress, responses that may lead to the sacrifice of many individuals, but that may increase the survival of the population, are activated (Storz and Hengge-Aronis, 2000).

Many kinds of aromatic compounds are generally regarded as extremly toxic to living organism and therefore impose the environmental pollutants as stress in the contanminated environment. It is accepted that the accumulation of aromatic compounds in the membrane is the main mechanism for toxicity, although other mechanisms can not be ruled out because of specific chemical properties of each specific compound (Sikkema *et al.*, 1995). The accummulation of the aromatics results in the disruption of the membrane structure and bilayer stability by increasing membrane fluidity. These adverse effects on the membrane are known to cause the loss of membrane functions, leading to cell death (Kieboom and De Bont.

2000).

Such specific stress responses can be provoked under the specific environmental conditions to cope with the specific stress situation. One of them is induction of proteins that eliminate the stress agent or repair cellular damage produced by the stress. These responses allow the cell to continue to grow, or at least survive, under the specific stress conditions. Others are general stress responses, such as the sigma-S and sigma-B regulated responses. Such responses can be governed by a single or a few crucial master regulator (Storz and Hengge-Aronis, 2000).

A general picture of how cells respond to stress is emerging, as shown in Fig. 1, although our knowledge is still fragmentary in many cases. In general, the response to stress is transitory. A shift in an environmental parameter is first detected by the cells, which subsequently adjusts gene expression to counteract the physical effects of the external change on its molecular components, finally recovering a steady state. Sensing mechanisms are not well understood. A transcriptional response follows in such a way that a set of specific proteins is synthesized. In this process (Fig. 1), the signal transduction between the sensor and the transcriptional machinery is not fully understood. At any rate, the situation is complex and possibly a cross-talk among different signaling mechanisms exists (Garcia and Forterre, 2000).

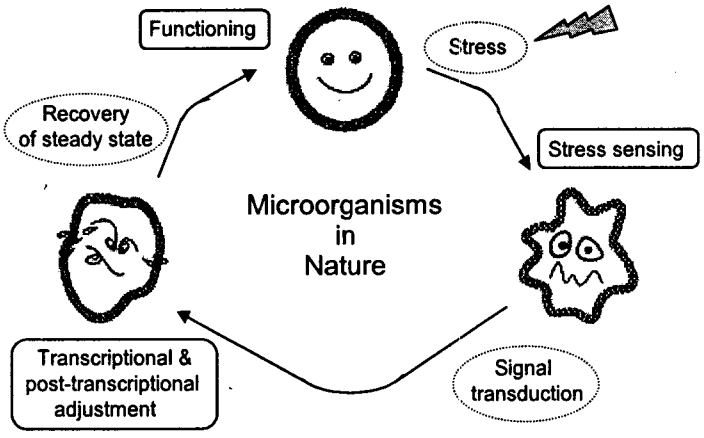

Fig. 1. General mechanisms involved in the transient response to environmental stresses.

Aromatic pollutants including halogenated and polycyclic aromatics have been reported to disrupt the endocrine hormone systems of higher organisms (Blasco *et al.*, 1997). The lipophilic properties of the pollutants are known to affect the cytoplasmic membrane (Sikkema *et al.* 1995). Aromatic hydrocarbons, such as sodium salicylate, benzene, and pentachlorophenol, have been reported to induce the stress shock proteins in bacteria (Faber *et al.*, 1993; Van Dyk *et al.*, 1995). Blom *et al.* (1992) reported that exposure of *E. coli* to nine different model micropollutants led to the induction of 13 to 39 stress shock proteins. However, some limitted studies of stress responses have been made with *Pseudomonas putida* for responses to heat shock and starvation stress (Givskov *et al.*, 1994; Lupi *et al.*, 1995; Selifonova *et al.*, 1996), but not for responses to chemical stresses. In *Pseudomonas* spp. the stress shock proteins and tolerant responses were reported to be induced by BTEX aromatics (Applegate *et al.*, 1998; VanBogelen *et al.*, 1987) and toluene (Vercellone-Smith and Herson, 1997).

Pseudomonas sp. DJ-12 is an aerobic degrader of 4-chlorobiphenyl (4CB) and biphenyl. The organism is capable of transforming 4CB to produce 4-chlorobenzoate (4CBA) and degrading 4CBA to 4-hydroxybenzoate (4HBA) (Han *et al.*, 1993; Chae and Kim, 1997). The production of stress shock proteins has been studied in *Pseudomonas* sp. DJ-12 cells treated with several aromatic hydrocarbons (Park *et al.*, 1998a; 1998b; Ko *et al.*, 1999). In this study, therefore, we report the degradative and protective functions of *Pseudomonas* sp. DJ-12 cells in terms of survival, change of morphology, production of stress shock proteins, and tolerance to the lethal concentrations, when treated with several pollutant aromatics at various concentrations.

II. Responses of *Pseudomonas* sp. DJ-12 to aromatic pollutants in degradability, viability, and morphology

Pseudomonas sp. DJ-12 is a degrader of biphenyl, 4CB, and 4HBA at 0.1 to 5 mM concentrations as reported by Han *et al.* (1993). The degradability of the strain DJ-12 with regards to higher concentrations of the aromatics is shown in Table 1.

Table 1. Degradation of aromatic hydrocarbons by *Pseudomonas* sp. DJ-12

Aromatics	Incubation time (hour)	Degradation at different concentrations (mM); 3	5	7	10
Biphenyl	6	−	−	−	−
	18	+++	+++	+	−
	30	+++	+++	++	−
4-Chlorobiphenyl	6	−	−	−	−
	18	+	+	−	−
	30	++	+	−	−
4-Hydroxybenzoate	1	−	−	−	−
	6	+++	++	−	−
	18	+++	+++	++	−
	30	+++	+++	+++	−

+++, 80% degradation of the initial amount of the aromatics; ++, 60% degradation; +, 40% degradation; −, no degradation.

Biphenyl and 4CB at 3 to 7 mM concentrations were degraded by the organism when incubated for 18 hours. Biphenyl was degraded more easily than 4CB, which was degraded by only 60% of its initial concentration (3 mM) after 30 hours incubation. On the other hand, 4HBA at 3 to 7 mM concentrations was rapidly degraded even after 6 hours. However, the higher concentrations of 4CB (7 mM), and biphenyl and 4HBA (10 mM) were not degraded even after 30 hours incubation. 4HBA is a metabolite compound produced from biphenyl via *meta*-ring cleavage, and can be further degraded by *Pseudomonas* sp. DJ-12 (Nam *et al.*, 1994). The dechlorination as reported by Chae and Kim (1997). Therefore, the degradation order of the three aromatics tested by this organism is theoretically rational on the basis of their chemical structures.

The survival of *Pseudomonas* sp. DJ-12 treated with each aromatic at various concentrations in MM2 medium are shown in Fig. 2. In general, the survival rate of the organism during incubation for 6 hours was reversely proportional to the concentration of each aromatic. The viability of the organism was not affected by the aromatics at lower concentrations: that is, biphenyl at 10 mM or less, 4CB at 30 mM or less, and 4HBA at 7 mM or less. The survival at 50 and 70 mM concentrations of biphenyl was decreased by 2 and 4 logs, respectively, after 6 hours incubation, and the survival at 60 mM of 4CB decreased by less than 2 logs (Fig. 2).

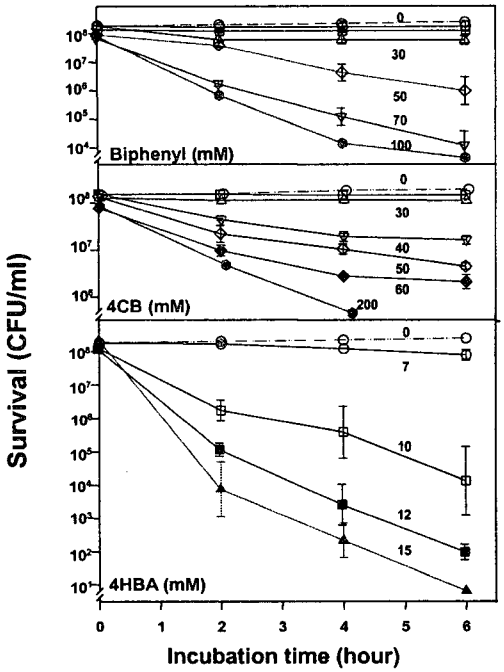

Fig. 2. Survival of *Pseudomonas* sp. DJ-12 in MM2 broth containing various concentrations of biphenyl, 4-chlorobiphenyl, and 4-HBA.

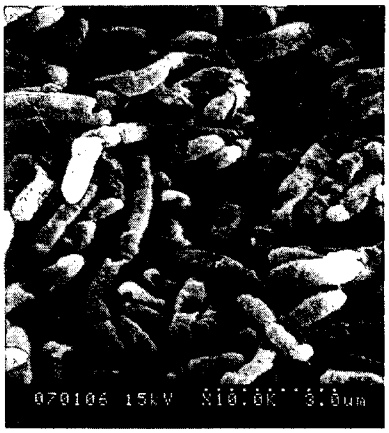

Fig. 3. Scaning electron mirograph of *Pseudomonas* sp. DJ-12 treated with 4CB (60 mM, 1h).

However, the adverse effect of 4HBA on viability was much more drastic. 4HBA (10 mM) reduced the survival rate by about 4 logs after 6 hours incubation and 15 mM by more than 7 logs (Fig. 2). Such a difference among three aromatics in the effect on viability of strain DJ-12 was positively correlated to the difference in degradation of the aromatics shown in Table 1. That is, the water-soluble 4HBA of higher biodegradability showed a greater killing effect than biphenyl and 4CB at the same concentration.

A scanning electron micrograph of the cells exported to 4CB for 1 hour are shown in Fig. 3. Normal cells exhibit a typical rod shape with smooth surfaces, but the morphology of the treated cells showed some destructive openings on the cell envelopes and irregular rod forms with wrinkled surfaces. Those cells treated with 7 mM 4CBA also indicated some

destruction 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.* (1992). The cell membranes of *Pseudomonas putida* were equally destroyed by exposure to toluene and xylene (Bollag *et al.*, 1996).

III. Production of stress shock proteins and development of tolerance induced by aromatic pollutants

Production of the stress protein in *Pseudomonas* sp. DJ-12 by treatment with the aromatic pollutants at various concentrations was studied as shown in Fig. 4. The induction of the stress protein which specifically reacted with anti-DnaK antibody was increased in those cells treated with biphenyl at 3 mM or higher concentration for 30 min or longer. However, DnaK protein was produced in control cells. This is reason why the heat-shock protein, DnaK, is produced even at normal temperatures (about 1.4% of the cellular protein) as reported by Gomes *et al.* (1990).

DnaK stress protein was also produced when the organism was treated with 4CB at 30 mM or higher concentration for longer than 20 minutes (Fig. 4.). The stress protein was more extensively induced by stress of soluble 4HBA. It was produced in cells treated with 4HBA at 3 mM or higher concentration for only 10 minutes. However, the protein was not detected in cells treated with 3 to 7 mM 4HBA for 60 minutes. In this study with *Pseudomonas* sp. DJ-12, the stress protein analyzed with anti-DnaK antibody was commonly produced by the three aromatic pollutants.

The cells of strain DJ-12 in which stress proteins had been produced by treatment with each aromatic pollutant exhibited a certain degree of tolerance to higher concentrations of the same aromatics as shown in Fig. 5. The soluble 4HBA showed the greatest tolerance induction and most distinct effects in survival and stress protein production. It is well known that a variety of cell types have the capacity to adapt adapting to normally lethal conditions after short pretreatment to sublethal conditions (Neidhardt and Van Bogelen, 1987). Blom *et al.* (1992) reported that chemical stress proteins were synthesized even at concentrations that had no effect on growth. Such a response was also observed in this study. 5 mM biphenyl, 30

mM 4CB, and 5 mM 4HBA did not affect the survival rate of DJ-12 strain. However, the aromatics at these same concentrations induced some tolerance to higher concentration of each aromatic.

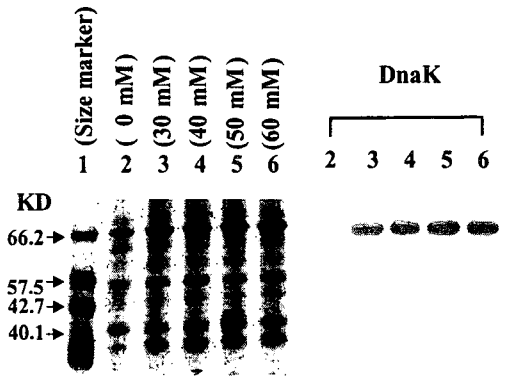

Fig. 4. SDS-PAGE (A) and Western blot (B) analyzed with anti-DnaK monoclonal antibody for the proteins induced by 4-chlorobiphenyl.

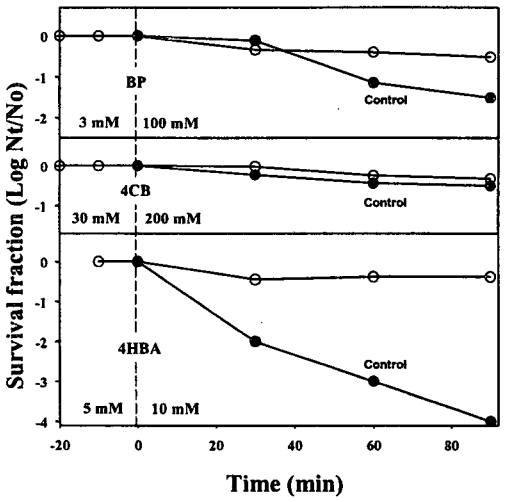

Fig. 5. Survival tolerance of the acclimatized *Pseudomonas* sp. DJ-12 to lethal concentrations of the same stresses.

IV. Protective function of the adjusted cells to aromatic pollutants, and its applications

Pseudomonas sp. DJ-12 is capable of degrading biphenyl, 4CB, and 4HBA at relatively low concentrations. In the case of microbial responses to toxic aromatic pollutants, a few papers have been reported so far. Nothing has been elucidated for the molecular functions in the sensing mechanisms of the aromatic pollutants and signal transduction of the chemical stress caused by aromatic pollutants. Here, we have studied some phenotypic responses of a *Pseudomonas* strain to several toxic aromatics in order to understand how to adapt to minor stress of the aromatic pollutants and how to protect themselves against the lethal stress. In this study, the survival of the cells, production of stress protein, and induction of tolerance

were examined by exposing to various concentrations of the aromatics. The viability of the organism was reversely proportional to the increasing concentration of each aromatics during 6 hours incubation.

Cells of the strain DJ-12 acclimatized by the stresses of biphenyl, 4CB, 4HBA, ethanol, and heat were then subjected to lethal conditions of each stress in order to examine the cross protection against different stresses. Several stress shock proteins including DnaK and GroEL were synthesized in the cells treated with each stress at sublethal conditions. Some of them were commonly produced by different stresses. The cells adapted to each stress under the sublethal conditions exhibited functioning of increased protection to the same stress as well as other stresses at lethal concentrations. However, their degradability of the aromatics was decreased. In particular, the cells pretreated with water-soluble 4HBA showed the greatest protective function for survival than those adapted by stresses of insoluble aromatics.

Although much has been learned about the bacterial responses to other stresses, we are far from a complete picture. For most responses, it is not clear how the initial signal is perceived. It is also becoming increasingly apparent that the different stress responses are extensively interconnected. Many target genes are still unknown, and the molecular functions of the target genes are still ambiguous. The physiological outcomes of many stress responses are not yet well understood. Future studies need to view the functioning of the cell as a complex regulatory network (Storz and Hengge-Aronis, 2000).

Early application used bacterial cell viability as test for toxicity. The Microtox assay described first in 1977 relies on bioluminescence, a rapidly generated and easily quantified signal (Bulich, 1982). But, this test is not predisposed to identification of the stress that a cell encountered. Further technological advances involved the construction of gene fusions; such fusions allow convenient, though indirect, measurements of specific mRNA species. The genes and operons used for fusion to promoters are *E. coli lacZ*, *P. putida xylE*, *E. coli phoA*, *galK*, *uidA*, *cat*, *nptII*, insect *luc*, *luxAB*, *luxCDABE*, and coelenterate *gfp* (LaRossa and Van Dyk, 2000). Besides the promoters of the specific genes, such as *pbr* regulatory genes, *xyl* catabolizing genes, *grp* heat shock genes, and many others, promoters of bacterial stress shock genes and the genes responsible for biodegradation of toxic aromatic pollutants working as environmental stress can be also used for fusion to the reporter genes.

It is well known that microorganisms integrate a wealth of information as they tune themselves to perform optimally in their environments. Therefore, the knowledge of stimulons and regulons as a master regulatory gene must be accumulated to identify all genes to be sensed by a single environmental challenge, and then those information of the genes and response mechanisms to stress can be utilized for monitoring of the toxic pollutants.

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V. References

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