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Toxicity Assays and Naphthalene Utilization by Natural Bacteria Selected in Marine Environments

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Polycyclic aromatic hydrocarbons (PAHs) exhibit toxic, carcinogenic or teratogenic properties (Saethre et al. 1984). PAHs exhibit very low solubility in aqueous solutions and are often associated to particle surfaces. For this reason their environmental distribution is mildly affected by natural processes, such as volatilization, photolysis and biodegradation, making PAHs to be preserved under many natural conditions. Their toxicity coupled with their chemical persistence makes these compound very dangerous as enviromnental contaminants. The presence of PAHs in marine environments is mainly anthropogenic, given that they are present in crude oil and are chronically released into the environment directly or after chemical manipulation like combustion processes.

Toxicity assay of PAHs compounds is a fundamental parameter to evaluate their effect on the marine biota. In the microbiota, toxicity is usually measured in terms of growth inhibition, oxygen consumption and enzyme activity (Liu et al. 1989). Microbial toxicity tests results are generally in good correlation with the ability to use aromatic hydrocarbons as carbon source and to degrade and mineralize a suit of petroleum hydrocarbons. The results of several studies have indicated that marine and freshwater microbiota inhabit sediments from relatively contaminant-free environments have the ability to degrade some PAH compounds (Herbes and Schwall. 1978). However, microbial populations can often adapt to transform specific compounds more rapidly as a result of prior exposure to a PAH mixture (Spain and Van Veld. 1983).

The present study was carried out to evaluate the toxic effects of naphthalene on marine bacterial strains collected from a relatively pristine environment and to compare these results with those obtained when strains are sampled from a PAH contaminated area. Furthermore, the ability to degrade naphthalene and to use it as sole source of carbon was tested for each of the strains studied.

MATERIALS AND METHODS.

Sediments and water samples were obtained from two different aquatic environments: (1) Morrocoy National Park, a pristine, protected coastal environment. Three different communities were sampled: Thalassia, Mangrove and Coral Reef. (2) A sandy beach subjected to runoff and other discharges from adjacent oil-refinery and others industries.

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Salinity, temperature, pH and Total Residual Polycyclic Hydrocarbons (TRPH) from water and sediments (collected from 0 to 1 cm. of depth) were analyzed according to Standard Methods (APHA. 1992). Bacterial communities were obtained according Clesceri el al. 1992. Marine mineral medium (MM) was prepared according to Geraldt el al. (1994), supplemented with 24g/L NaCl. Nutritive medium (NM) was composed of marine medium supplemented with nutrient broth (Difco), 10g/L triptose, 3g/L casein hydroxilate. All culture medium pH was adjusted to 7.0.

For the isolation of naphthalene tolerant bacterial strains, one ml. (10% inoculum) of sediment or water was inoculated into the liquid nutritive media (NM) previously dosed with (1, 10 and 100 mg/L naphthalene) dissolved in 1% dimethilsulfoxide (DMSO). The effect of various concentrations of DMSO on the bacterial growth was previously tested and was negligible compared with lowest naphthalene treatment. The bacterial suspensions were incubated in the darkness at 27°C during 24hr. Strains tolerant to the highness naphthalene concentration were isolated and purified on solid medium. The numbers were determined from the colony forming units (CFU). Morphological and physio-biochemical characteristics of the tolerant strains were determined according to standard descriptions of Bergey's (1994).

Inoculum for naphthalene toxicity assays were prepared from pure culture, grown for 24 hours in nutritive medium at 27 °C, and shaken at 140 rpm. Flasks with nutritive medium containing various concentrations of naphthalene (50, 100,250,500 and 1000 mg/L) dissolved in DMSO, were inoculated. Growth rate was determined for a period of 12 hr. by measuring the optical density at 600nm according to Gerhard et al. (1994). Controls containing only DMSO were also determined by measuring the optical density. In addition, colony forming units (CFU) were simultaneously determined every hour on nutrient agar plates. All treatments were done in triplicate. As in the previous experiment, samples were maintained in water bath at 27°C, shacked in the darkness at 140 rpm. The naphthalene concentration that inhibits 50% of bacterial growth (IC50) was calculated by comparing the growth of the untreated controls versus the results of the CFU. The statistical treatment of the data was do using single-Anova analysis of variance. Significance levels were set at $P<0.05$.

Strains capable of utilizing naphthalene as sole carbon source were obtained by growing isolates in NM for 24 hr. at 27°C. One ml. of the bacterial suspension was centrifuged and the pellet was transferred to MM supplemented with naphthalene at increasing concentrations (0.1, 1, 10 mg/L and naphthalene crystals). Naphthalene was supplied by placing the compound at the top of the plate, according to Wodizinski and Bertolini. (1972). Every 10 days, duplicate subsamples from each treatment were used for the CFU count. The tests were carried on for 30 days. All the treatments were incubated in the darkness to avoid photooxidation.

Preliminary naphthalene degradation test was carried out in NM containing three naphthalene concentrations (50, 500 and 1000 mg/L). Flasks were inoculated as described before and kept in shaking bath (140 rpm) at 27°C in the darkness for 12 hours. The naphthalene remaining after this treatment was extracted with methylene chloride, according to Clescier et al. (1992). and analyzed by Gas Chromatography (HP 5890 connected to a Data integrator). The unit is equipped with a split injector and flame ionization detector (FID). Chlorobenzene was employed as internal standard. All treatments were done in triplicates, and controls were run to account for any volatilization of naphthalene.

RESULTS AND DISCUSSION

Results of the analysis of TRPH and physicochemical parameters of the water and sediments samples are shown in the Table 1. Parameters as temperature, pH and salinity exhibit very similar values for all the samples. As expected, total TRPH content show significant variations between the different communities; the higher value was found in the sandy beach in Golfo Triste. Previous analysis have revealed the presence of up to 313 ppm of TRPH in the sediments of this locality (Corpoven. 1995). By it's proximity to the industrial park (oil refinery) and others different sources of contamination, this coastal zone is considered a chronically polluted area.

On the other hand, the sediments Thalassia (Morrocoy National Park) also contained high TRPH concentration. This community is located near to a gasoline boat supply and this may account for intermittent contamination by fuel and oil residues. No detectable concentrations of TRPH's was found in water samples.

The bacterial screening was done in mixed culture supplemented with different naphthalene concentrations. Three tolerant strain isolates were selected at a concentration of 100mg/L of naphthalene. In all samples, total number of tolerant organisms was around 10^{7} CFU/mL. Morphological and physio-biochemical characteristics of the isolates are shown in Table 2. Although, according to the standard description in Bergey's manual of Determinative Bacteriology (1994), isolates IS1, IS2 and IS3 were identified respectively *as* genus *Aeromonas, Sthaphylococus* and *Aereobacterium, this* identification will need more detailed studies for confirmation.

The toxic effect of naphthalene on the bacterial growth for the short-term assays is shown in Fig. 1. In addition, the isolated strains were tested for their tolerance to naphthalene concentrations higher than those used in mixed culture. All three isolates showed different degrees of resistance to naphthalene in the 12-hr assay as follows: (1) IS1 growth rate is significantly affected by naphthalene concentration at 100 mg/L ($P < 0.005$),. The $IC_{50\%}$ for the isolate was determinate to be at 500 mg/L. (2) IS2 growth rate was suppressed at a concentration of 50 mg/L. The $IC_{50\%}$ for this strain is 100 mg/L. A bactericidal effect was also observed at 500 mg/L and up. A longer lag phase was detected when the highest concentration of naphthalene (1000mg/L) was used. (3) IS3 growth rate is, in contrast, affected at 100 mg/L, but the $IC_{50\%}$ was attained at the highest concentration tested (1000mg/L) without any drastic inhibition at the end of the growth.

These results suggest that the ability of the isolated bacteria to tolerate the toxic effect of naphthalene is related to a pre-exposure of the microbial communities to PAH in their natural environment. Microbial communities from chronic pollutant exposure sites (IS1) tolerate higher naphthalene concentrations than microbial communities from pollutant-free environment (IS2).

The enhanced tolerance to hydrocarbons after exposure in aquatic environments could be explained partly by differences in the inn-a-and extracellular mechanisms induced by direct response to PAH's (enzymatic system, bioemulsifier secretes, etc). This may be the major contributing factor to the limited naphthalene toxic effect observed for isolate IS3.

Table 1. Physical and chemicals parameters of the sites used in this study.

Locality	Temperature (°C)	Salinity (ppm)	pH	TRPH Sample Water (ppm)	TRPH Sample Sediments (ppm)
Sandy Beach	28	36	7.8-8.3	<15	54
Thalassia	30	38	7.5-8.0	<15	47
Coral Reef	29	38	7.5-8.0	<15	14.2
Mangroves	29	39	7.5-8.0	<15	<15

Values are presented as mean of duplicates.

Table 2. Morphological and biochemical characteristics of selected naphthalene-tolerant bacterial isolates.

Character	IS1	IS2	IS3
Locality (community)	Thalassia	Mangrove	Sandy beach
Colony Color	Creamy	White	Yellow
Gram reaction	-	+	+
Morphology	Rod	Cocci	Rod
Motility	+	-	-
Respiration	Facultative anaerobic	Facultative anaerobic	Facultative anaerobic
Catalase	+	+	+
Oxidase	+	+ weak	+ weak
Indol	-	-	-
RM	-	-	-
VP	+	+	+
Nitrate reduction	+	+	+
Citrate	+	-	-
Urease	-	+	-
Glucose	+	+	-
Lactose	+	+	-
Growth in presence of 36%NaCl .	+	+	+

Several lines of evidence suggest that PAH's biodegradation in microbial populations from aquatic environments is a direct result from the adaptation or acclimation of a few natural bacterial (Wiggins. et al. 1987; Bauer and Capone. 1988), and that the rate of biodegradation depends on both, pre-exposure time and concentration (Spain et al. 1980, 1983).

As tolerance is frequently linked with the ability of an organism to metabolize the toxic compound all three strains were tested for their capacity to growth in presence of naphthalene as their sole carbon source. The results show that isolates IS1 and IS3 grow at expense of naphthalene at concentrations up to 10 mg/L, during 30 days (Fig.2). However, in the same conditions, isolate IS2 cannot utilize naphthalene as sole carbon source. The growth rate for IS1 and IS3, compared to the controls (MM only), is significantly high. These and other similar results (Shuttleworth and Cerniglia. 1996) strongly suggest that exposure to PHA's in natural environment permit to microbial

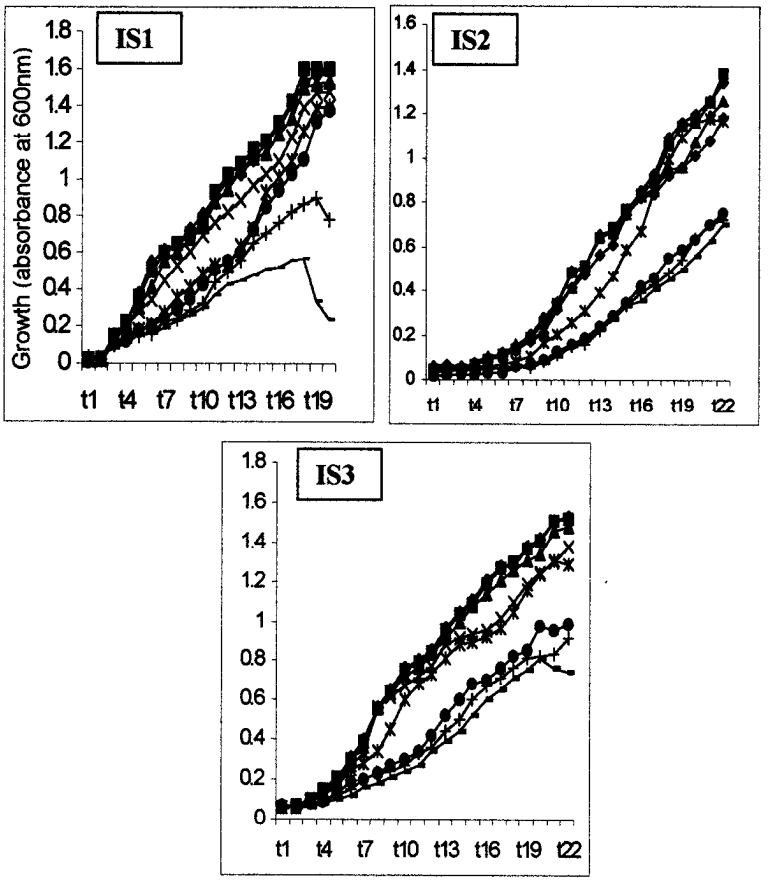

Figure 1. Growth curves for IS1, IS2 and IS3 exposed to different naphthalene concentrations. t=hr.◆Control (NM +bacteria.).■ NM+b+DMSO.▲50 ppm. X 100 ppm✳ 250 ppm.● 500 ppm. ▮750 ppm. – 1000 ppm. Data are mean of triplicates

populations the necessary affinity for these compounds to be able to use them as sole carbon source.

The importance of this exposure in natural environment is underlined by the fact that, of the all three tolerant strains selected only the one originating from the pristine environment was unable to use naphthalene as the sole source of carbon.

The results of naphthalene degradation experiments are shown in Fig.3. All three strains were capable to degrade low concentrations (50 mg/L) of naphthalene in supplemented nutritive marine medium within 12 hr. At this concentration, no drastic changes in the growth curves were detected for any of the strains in the toxicity assay. Given that isolates IS1, IS2 and IS3 were selected for their tolerance to naphthalene, it is not surprising that all three were able to degrade small amounts of the compound. Furthermore, it is very likely that one of the most important requirements for the tolerance behavior in all three strains involves the ability to degrade naphthalene even at low concentration (Spain et al. 1980;1983; Bauer and Capone. 1988).

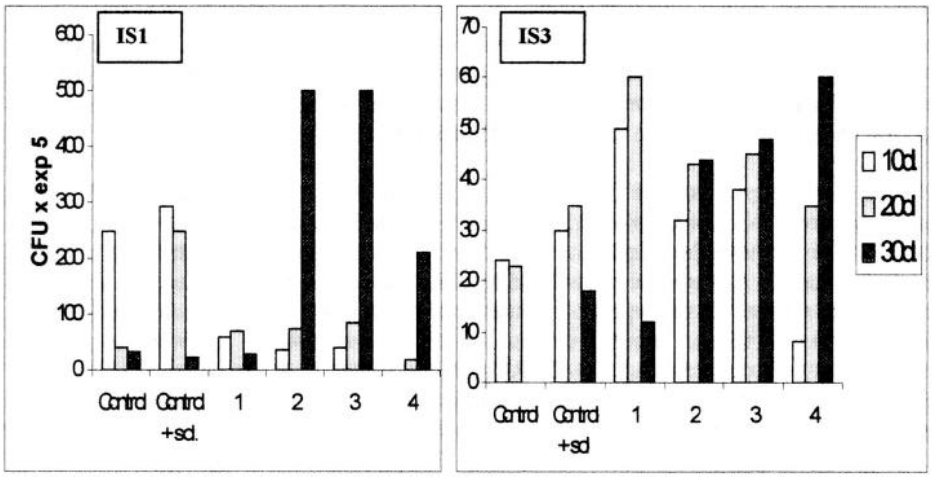

Figure 2. CFU in basal medium supplemented with naphthalene as sole carbon source. Treatments: Control + sol (Medium +b+ DMSO). 1 (0.1 mg/L) 2. (1 mg/L) 3. (10mg/L) 4. (Naphthalene crystals). Data are the mean of two replicates.

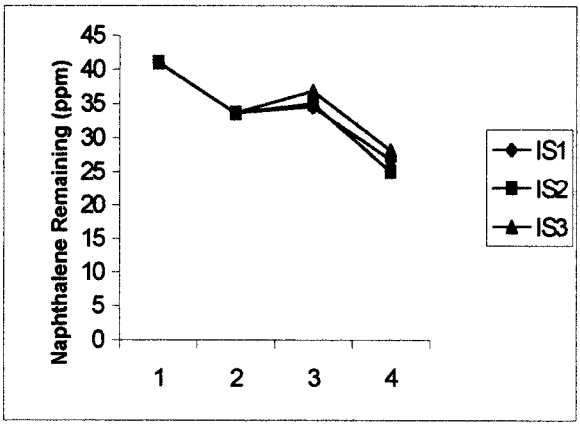

Figure 3. Naphthalene biodegradation assay at 50 ppm. Control (NM+naphthalene). 2.Evaporation control (NM+naphthalene).3. T1=(NM + naphthalene + bacteria) (0 hr). 4.Tf =(NM + naphthalene + bacteria) (12 hr. after). Data are mean of duplicate determinations (n=2)

It has often been suggested that acclimation in polluted environments, reflect the presence of induction of enzyme systems necessary to degrade the compound of interest. The induction of this system results in the degradation or biotransformation of xenobiotic compounds (Wiggings et al. 1987). It cannot be inferred from our results what particular mechanism (enzyme induction, NAH + plasmids, etc) is responsible of the bacterial tolerance to the toxic effects of naphthalene. However, the data suggest a good correlation between tolerance mechanisms and the degree of pre-exposure to the pollutant in the natural environment.

From our results we may conclude that: 1.populations of bacteria from PAH's polluted sediments (IS1 and IS3) show greater adaptation response (increased toxic tolerance, sustained growth) to the toxic effects of naphthalene than populations from less polluted environments (i.e. IS2). 2.the frequency of the exposure to the toxic compound in the natural environment seems to determine different degrees of acclimation.

Historically, the presence of PAHs in natural environments has been attributed to two main sources of contamination: biogenic or anthropogenic. Over eons, many microorganisms have developed mechanism to tolerate and even exploit these naturally occurring hazard as potential growth substrates. During the last decades, oil spills and many other sources of anthropogenic contamination have increased the selective pressure on bacteria, algae and fungal strains towards those able to growth in polluted environments. The understandings of PAHs biodegradability, as well as the nature of the biodegradation processes are of key importance in order to design effective bioremediation strategies in polluted environment.

The present work is a first step in the understanding of the response of the microbial communities from the marine sediments to PAHs contamination in the Venezuelan coast. These results may contribute to the understanding of the adaptative response of tropical bacteria to hydrocarbon contamination, and may help to provide an interesting model for future research in tropical environment.

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