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Hydrocarbon Degrading Bacteria from Pakistani Soil: Isolation, Identification, Screening and Genetical Studies

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Abstract: Sixty bacterial strains (isolated from soil near different petrol pumps of Karachi city) were screened for hydrocarbon degradation. They were identified on the basis of morpho-cultural and biochemical characteristics and were found belonging to different genera including *Staphylococcus*, *Corynebacterium*, *Bacillus*, *Proteus*, *Pseudomonas*, *Klebsiella* and *Escherichia*. Four solid hydrocarbons including biphenyl, camphor, phenanthrene and naphthalene and four liquid hydrocarbons i.e. benzene, toluene, octane and heptane were used in this study. In the case of solid hydrocarbons a total of 17% degraded biphenyl, 8% degraded camphor, 8% degraded naphthalene and 10% degraded phenanthrene. In case of liquid hydrocarbons about 57% were able to degraded benzene, 55% degraded toluene, 48% degraded octane and 33% degraded heptane. About 89% isolates degraded both solid and liquid hydrocarbons while 11% could not degrade any of them. In order to locate the genes responsible for hydrocarbon degradation, curing experiments were performed using physical agent i.e. elevated temperature and the chemical agent i.e. acridine orange. Results of these experiments indicated that the genes are plasmid born. *In vivo* direct gene transfer (bacterial conjugation) experiments were also performed and it was shown that hydrocarbon-degrading plasmid genes are stably transferable to the competent recipient cells.

Key words: Hydrocarbon degradation, solid-liquid hydrocarbons, curing, conjugation

INTRODUCTION

Organic compounds are broadly divided into three classes namely aliphatic, salicylic and aromatics. The basic structural unit of aromatic compound is benzene (C_6H_6). Aromatic hydrocarbons are insoluble in water but soluble in non-polar organic solvents. Aromatic and heterocyclic compounds include anthracene, phenanthrene, naphthalene, biphenyl, camphor, toluene, fluorine, benzene, thianthrene, dibenzothiophene, carbazole, dibenzefuran, indole, quinoline and xylene[1]. Polycyclic aromatic hydrocarbons are the proven environmental carcinogens. They causes cytotoxicity, DNA adduct formation and induction of mutation[2]. Exposure with hydrocarbons (such as benzene and naphthalene) takes place through air and breathing resulting in lungs and heart disorders[3]. Polyaromatic hydrocarbons arise from incomplete combustion of organic matter inflames, engine and high temperature industrial processes[4]. Biodegradation is a process by which a potentially toxic compound is converted into a less/non-toxic compounds by metabolic activities of microorganisms that utilize the pollutant as the source of carbon and energy[5]. The ability of soil microorganisms (particularly pseudomonads) to metabolize biphenyls (Bps) and poly chlorinated biphenyls (PCBs) is well known[6]. The genes that encode enzymes to degrade some Bps were chromosomal as well as plasmid-borne[7]. Hydrocarbons contaminated soil is classified as hazardous waste and is toxic to microorganisms as well as to the plants[8]. A large number of hydrocarbon degrading bacteria (including the gram-positive and gram-negative with particular reference to *Pseudomonas* spp.) have been identified from oil-contaminated soil. Other includes *Rhodococcus* and *Acinetobacter* spp.[9]. The *Staphlycoccus* spp. have ability to degrade the base oil. The organism produces heavy growth in the mineral salt, base oil and agar media[10].

MATERIALS AND METHODS

Bacterial strains and media: Sixty bacterial strains (isolated from soil samples of different petrol pumps of Karachi) were identified on the bases of morphological, cultural and biochemical characteristics and used for hydrocarbon degradation studies. Nutrient agar (NA), mineral salt agar (MM2) and basal salt agar (BSA) were used for screening of cultures for hydrocarbon

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degradation. Brain heart infusion (BHI) medium was used for the plasmid gene(s) curing experiments.

Chemicals: Solid hydrocarbons used in this study are biphenyl, camphor, nepthalene, phenanthrene (dissolved in ether) and liquid hydrocarbons include benzene, octane, heptane and toluene (Merck, Germany).

Assay for solid hydrocarbon degradation: Spray plate technique[11] was followed for this purpose. The overnight cultures were spotted on MM_2 agar plates (with or without glucose) by replica plate technique. Hydrocarbon solutions (10%) were prepared in ether and sprayed on to the surface of the MM2 agar plate. The ether was evaporated leaving behind a thin film of hydrocarbon on the agar surface. The plates were incubated at 37°C for 3-4 days. The appearance of growth on the plates indicated positive tests.

Assay for liquid hydrocarbon degradation: In order to monitor the liquid hydrocarbon degradation the overnight cultures were spotted on basal salt medium (pH 7.0) by replica plate technique and then wells were made (in the periphery) and loaded with hydrocarbon soaked cotton. Controls were also run (i.e. basal salt medium with and without glucose). The plates were incubated at 37°C for 3-4 days. The appearance of growth on the plates indicated positive test[12].

Curing (Plasmid elimination): The curing experiments were performed using both physical (elevated temperature)[13] and chemical (acridine orange)[14] agents. Conjugation (in *vivo* direct gene transfer): Donor and recipient cultures were grown separately in BHI broth at 37°C for 24 h. Next day both the cultures were inoculated in fresh BHI broth separately and incubated in shaking water bath at 37°C for 2-4 h. Donor and the recipient cultures were mixed in 1:10 ratio and incubated again for 24 h at 37°C. Tube contents were then centrifuged (4000 rpm for 20 min), supernatant discarded and a loopful of pellet was streaked onto selective medium for isolated colonies which were then tested for hydrocarbon degradation ability[15].

RESULTS AND DISCUSSION

Microorganisms play an important in the degradation of hydrocarbons. Degradation is accelerated due to chemically induced selection or adaptation of microorganisms resulting from previous exposure to chemicals (hydrocarbons). Polyaromatic hydrocarbons (PAHs) are wide spread environmental contaminants. In order to observe the ability of soil microorganisms to degrade solid and liquid hydrocarbons, sixty bacteria were isolated from soils collected from different petrol pumps of the cosmopolitan city of Karachi. They isolates were identified on the basis of morpho-cultural and biochemical characteristics and were found belonging to different gram-negative and gram-positive genera namely *Staphylococcus*, *Corynebacterium*, *Bacillus*, *Proteus*, *Pseudomonas*, *Klebsiella* and *Escherichia* (Table 1). All isolated strains were screened for the ability of utilizing solid hydrocarbons including biphenyl, camphor, naphthalene and phenanthrene and liquid hydrocarbons including benzene, toluene, octane and heptane. Isolates which produced zones on mineral salt agar (MM2) glucose deficient in case of solid hydrocarbons and the ones showed the presence of growth in basal salt medium supplemented with liquid hydrocarbons (without glucose) were considered to possess the hydrocarbon degradation potential. Analogous results were reported by other workers showing the degradation of solid hydrocarbons by microbial populations in oil contaminated soil[9]. The present results are also in agreement with the finding of Siddiqui and Adams[8] who observed the growth of bacteria on media (depleted of glucose) containing liquid hydrocarbons as sole source of carbon. Table 2 shows the percentage of bacteria degrading liquid hydrocarbons (benzene, toluene, octane and heptane) while bacterial percentage degrading solid hydrocarbons (biphenyl,

Table 1: Percentage of different gram-positive and gram-negative hydrocarbon degrading bacterias isolated from soil samples near selected petrol pumps of Karachi

Bacterias	Hydrocarbon degrading stains/ Total no of isolates	Percentage (%)
Gram-positive:		
Bacillus spp.	6/60	10.0
Corynebacterium spp.	3/60	5.0
Staphylococcus spp.	7/60	11.6
Gram-negative:		
Escherichia coli	20/60	33.3
Klebsiella spp.	6/60	10.0
Proteus spp.	13/60	21.6
Pseudomonas spp.	5/60	8.3

Table 2: Liquid hydrocarbons degradation by different soil isolates

Hydrocarbons	Number of degrading bacteria/ Total number of isolates	Percentage (%) of degrading bacteria
Liquid:		
Benzene	34/60	56.66
Toluene	33/60	55.00
Octane	29/60	48.33
Heptane	20/60	33.33
Solid:		
Biphenyl	10/60	16.66
Nepthalene	5/60	8.33
Camphor	5/60	8.33
Phenentherene	6/60	10.00

Table 3: Pre-and post-curing hydrocarbon degradation potential by elevated temperature (48°C)

	Pre–curing degradation ability				Post-curing degradation ability			
Isolates	Benzene	Toluene	Octane	Heptane	Benzene	Toluene	Octane	Heptane
E. coli SS03	+	+	+	-	-	-	+	-
B. subtilis SS09	+	+	+	+	+	+	+	+
B. subtilis SS10	+	+	+	+	-	-	+	+/-

+ = growth (not cured); - = no growth (cured); +/- = partially cured

Table 4: Pre- and post-curing hydrocarbon degradation potential by acridine orange

	Pre-curing degradation				Post-curing degradation			
Isolates	Benzene	Toluene	Octane	Heptane	Benzene	Toluene	Octane	Heptane
B. subtilis SS10	+	+	+	+	-	-	-	-
K. pneumoniae SS12	+	+	+	+	+	+/-	-	+
K. pneumoniae SS26	+	+	+	+	+/-	+/-	-	-
Ps. aeruginosa SS30	+	+	+	+	-	+/-	-	+/-
Pr. mirabilis SS31	+	+	+	-	-	-	-	-

+ = growth (not cured); - = no growth (cured); +/- = partially cured

Table 5: *In vivo* gene transfer between the octane and benzene degrading donor (D) and the non-octane and non-benzene degrading recipient (Re)

		Hydrocarbons degradation with selective markers	Hydrocarbons	
Cultures	Isolates	Antibiotic streptomycin (500 μg mL^{-1})	Benzene	Octane
Donor (D)	*E. coli* SS08	S	+	+
	E. coli SS03	S	+	+
	E. coli SS15	S	+	+
Recipient (Re)	*C. diphtheriae* SS50	R	-	-
	P. mirabilis SS21	R	-	-
Transconjugant (Tc)	D/Re			
	SS08/ SS50	R	+	+
	SS03/SS50	R	+	+
	SS15/SS21	R	+	+
	SS08/SS21	R	+	+

R=resistant, S=sensitive, D=donor, Re=recipient, Tc=transconjugant, + =hydrocarbon degrading strain, - =hydrocarbon non-degrading strain

anthracene, camphor and phenentherene). Figure 1 shows liquid hydrocarbon (benzene and toluene) degradation by different gram-positive and gram-negative soil isolates. In case of liquid hydrocarbons about 57% isolates were able to degraded benzene, 55% degraded toluene, 48% degraded octane while 33% degraded heptane. In the case of solid hydrocarbons, 17% isolates degraded biphenyl, 8% degraded camphor, 8% degraded naphthalene and 10% degraded phenentherene (Table 2)

In order to find out the location (chromosomal or extra chromosomal) of gene(s) responsible for the hydrocarbon degradation, plasmid curing experiments were performed using both physical and chemical agents. Elevated temperature mediated curing was undertaken with three selected isolates, which degraded the liquid hydrocarbons (Table 3). The marker hydrocarbons included benzene, toluene, octane and heptane. After curing, the ability to degrade hydrocarbons was eliminated in two of the isolates i.e. SS03 and SS10 while the isolate SS09 retained the ability to degrade the liquid hydrocarbon. It could thus, be concluded the genetic factor(s) responsible for degradation in this strain are chromosomal. After curing two isolates lost the ability to degrade benzene and toluene but retained the ability to degrade octane. Heptane factor was found fully cured in SS03 while partially cured in SS10. Acridine orange mediated curing was undertaken in five isolates (Table 4). Curing dose of acridine orange was worked out to be 0.2 mg mL^{-1}. Most of the cured isolates lost their ability to degrade liquid hydrocarbons including benzene, toluene, octane and heptane while some isolates were partially cured. Infect, the complete or partial curing is the function of copy number of the plasmids and the scattered distribution of the plasmid copies (the copies placed away from the periphery of the cell are not completely eliminated by the curing agent, because of accessibility range problem). Degradative (or resistant) determinants are often transposable and exist in both chromosomal and extra-chromosomal location[16]. It is likely that gene(s) for the hydrocarbon degradation in the third isolate were "transposable" (hence may be chromosomal at the time of exposure to curing agent).

Transfer of genes responsible for biodegradation of hydrocarbons plays an important role in bioremediation of pollutants[17]. Ability of isolates to transfer the hydrocarbon degradative plasmid gene (from donor to the

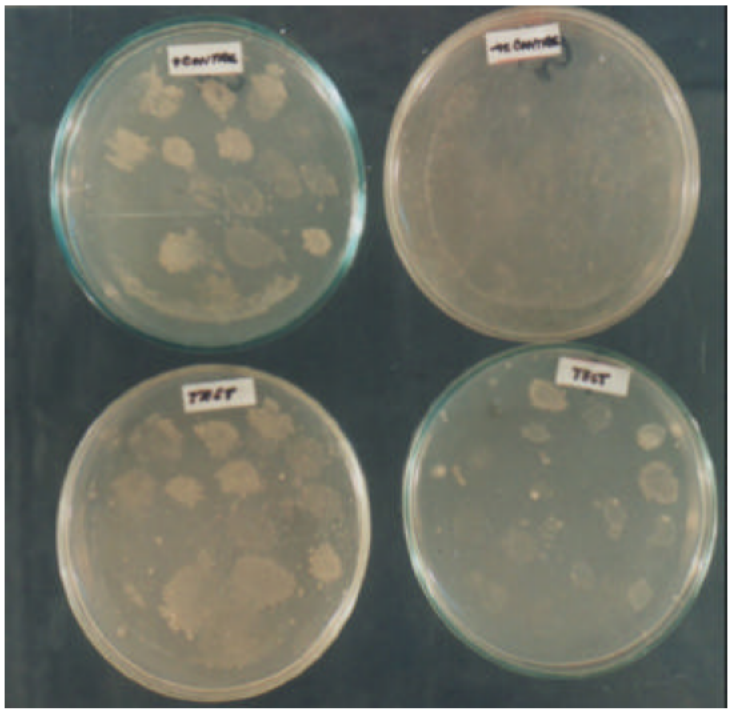

Fig. 1: Liquid hydrocarbon (toluene and benzene) degradation by different soil isolates. Top two plates= + control without glucose, -control with glucose Bottom two plates= Right supplemented with toluene, Left supplemented with benzene

recipient) is shown in Table 5. Isolates were also screened for drug resistance (for counter selection marker). Different representative isolates were used as the donor and the recipient strains. Transconjugants were counter selected on streptomycin containing media and checked for benzene and octane (liquid hydrocarbon) degradation. It was observed that hydrocarbon degradative genes were transferred from donor to the recipient strains. Horizontal transfer of benzene degrading genes was reported earlier[18].

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