Pak. J. Bot., 35(1): 37-44, 2003.

HYDROCARBON DEGRADATION BY MARINE BACTERIA: SCREENING AND GENETIC MANIPULATION

SHEIKH AJAZ RASOOL, SAMREEN IJAZ AND SYED ABDUS SUBHAN

Department of Microbiology,
University of Karachi, Karachi, Pakistan

Abstract

Forty marine bacterial isolates from Karachi coast were screened for hydrocarbon degradation. All the three solid hydrocarbons (biphenyl, naphthalene and phenanthrene) were degraded by 63% of the marine isolates, two were degraded by 25%, while one was degraded by 8% of these bacteria. The five liquid hydrocarbons (benzene, heptane, octane, toluene and xylene) were degraded by 13% of the marine isolates, while 27% isolates could degrade four hydrocarbons. About 10% marine isolates degraded all the solid and liquid hydrocarbons, whereas 3% could not utilize any form of hydrocarbon. Location of the genetic determinants responsible for degradation was determined by acridine orange mediated curing (plasmid elimination). Hydrocarbon degradation potential was lost after such curing thereby establishing the control of hydrocarbon degradation by plasmid-borne genes. These extrachromosomal plasmid-borne genes were stably transferred to the genuine competent and the cured (donor converted) recipient cells. Interestingly, the transconjugants also acquired the gene transfer potential to other prospective recipients.

Introduction

Pollution is considered as one of the most horrible ecocrises of the present time. Unregulated industrial and miscellaneous waste is discharged into marine enviroment which adversely affects the ecosytem (Odum, 1971). Oil leakage from heavy load of marine traffic and oil spills also contribute to the already deteriorating conditions (Ashraf & Jaffer, 1992). The megapoliton city of Karachi along with its refineries also contributes to the local oil pollution.

Degradation of crude oil, halogenated and aromatic hydrocarbons by microorganisms in sea, is an efficient mechanism of pollutant elimination (Frantz & Chakrabarty, 1986). Microbial hydrocarbon degradation is remarkably accomplished by variable accessory genetic elements (Finnerty & Attaway, 1992). The microorganisms utilize these compounds as sole source of carbon and energy. Infact, different pathways operate for degradation of short chain and halogenated hydrocarbons (Grund & Eaton, 1996). Polycyclic aromatic hydrocarbons (potentially carcinogenic) arise from incomplete combustion of organic matter inflames, engines and high temperature industrial processes (Ripp, 2000). These compounds constitute a major problem in bioremediation of pollutants due to extra low aqua solubility (Sutherland & Cerniglia, 1995). Polychlorinated biphenyls may enter aquatic enviroment as solvents, pesticides or deodorants. They are eliminated by volatization, photo and biodegradation. Some degradative gene segments have been transposable elements, which specify enzymes involved in the degradation of compounds like toluene and xylene (Chakrabarty *et al.*, 1978).

In view of the alarmful situation of hazardous marine ecopollutants, the present study was planned to base on the marine microbial degradation of hydrocarbons by:

1) Screening of marine isolates for hydrocarbon degradability.
2) Plasmid curing strategies for the location of degradation responsible genetic factors.
3) Transferability (modes and extent) behaviour of plasmid borne genetic factors (*in vivo* genetic manipulation).

Materials and Methods

Bacterial strains belonging to the genera of *Staphylococcus, Micrococcus* and *Pseudomonas* were obtained from Mr. Zaid Ahmad of LMG, Microbiology Department, University of Karachi.

Culture media: Zobell' s medium (broth and agar) was used for the propagation of marine bacterial cultures. Mineral salt agar (MM2) and basal salt medium were used for screening of cultures for hydrocarbon degradation.

Hydrocarbon sources and other chemicals: Solid hydrocarbons (biphenyle, naphthalene, phenanthrene; dissolved in ether) and liquid hydrocarbons (benzene, n-octane, toluene, xylene and n-heptane) were used in this study. Acridine orange (Sigma) was used for the purpose of plasmid elimination.

Hydrocarbon degradation was performed to determine the ability of the isolates to degrade (or reduce) the hydrocarbon compounds. Solid hydrocarbon degradability was assayed as per Kiyohara *et al.*, (1982); while liquid hydrocarbons were assayed as per Furukawa *et al.*, (1983).

Plasmid curing was performed as per Hirota (1960).

Plasmid conjugation was performed as per Miller (1972) to access the transferability of hydrocarbon degradation markers. The donor and the recipient marine cultures were grown separately in Zobell's broth at 20°C for overnight and 100μL of broth culture was transferred in 5mL broth separately in shaker and incubated at 20°C for 2-4 hours. Donor and the recipient were mixed in 1: 10 ratio and incubated for overnight at 20°C followed by centrifugation (3500 rpm for 20 minutes). Supernatant was discarded, followed by vortex for mixing. A loopful of this mix was streaked on Zobell's and the selective medium in order to select the transconjugants followed by stability assessment by replica.

Results

Figure 1 shows the solid hydrocarbon (nepthalene, phenanthrene, biphenyle) degradative potential of marine isolates while Table 1 illustrates the degradation data about liquid hydrocarbon (benzene, n-heptane, n-octane, toluene and xylene) by the marine isolates. Liquid (readily available) sources of hydrocarbons have been degraded by fewer isolates compared to the solid hydrocarbons. Table 2 presents the acridine orange mediated curing of selected hydrocarbon degradative potential by representative strains. Ability of marine isolates to transfer the naphthalene degradation potential is depicted in Table 3. Evidently, this plasmid genes based properties has been transferred in all the mating variables. Tables 4a and 4b show the results of retrotansfer of naphthalene degrading plasmid markers from transconjugants (used as donors) to the cured recipient cells. Almost all the experiments proved stably prospective.

Table 1. Hydrocarbon (liquid) degradation by marine isolates.

Isolate No.	Hydrocarbon				
	Benzene	Heptane	Octane	Toluene	Xylene
SI 1	+	-	+	+	+
SI 2	+	+	+	+	+
SI 3	-	+	+	-	+
SI 4	-	-	-	-	+
SI 5	-	-	-	-	-
SI 6	+	-	-	+	+
SI 7	+	-	-	+	+
SI 8	+	-	-	+	+
SI 9	+	-	+	+	+
SI 10	+	-	+	+	+
SI 11	-	-	-	-	-
SI 12	+	-	-	+	+
SI 13	-	-	+	+	-
SI 14	-	-	-	+	-
SI 15	-	-	+	+	-
SI 16	+	-	+	+	+
SI 17	+	+	-	+	+
SI 18	+	-	-	+	+
SI 19	+	+	+	+	-
SI 20	+	+	+	-	+
SI 21	-	+	+	+	+
SI 22	-	+	+	-	+
SI 23	-	-	+	+	+
SI 24	+	+	-	+	-
SI 25	-	-	+	-	-
SI 26	-	+	+	+	+
SI 27	+	-	+	-	+
SI 28	-	-	+	+	-
SI 29	-	+	+	+	+
SI 30	-	-	-	-	-
SI 31	+	+	+	+	+
SI 32	-	+	+	+	+
SI 33	+	+	+	+	+
SI 34	+	-	+	+	+
SI 35	-	+	-	-	-
SI 36	+	+	+	+	+
SI 37	-	+	+	+	+
SI 38	-	+	-	-	-
SI 39	-	+	+	-	+
SI 40	+	-	+	+	+

Keys: + Indicates hydrocarbon degrading strains.
- Indicates non-hydrocarbon degrading strains.

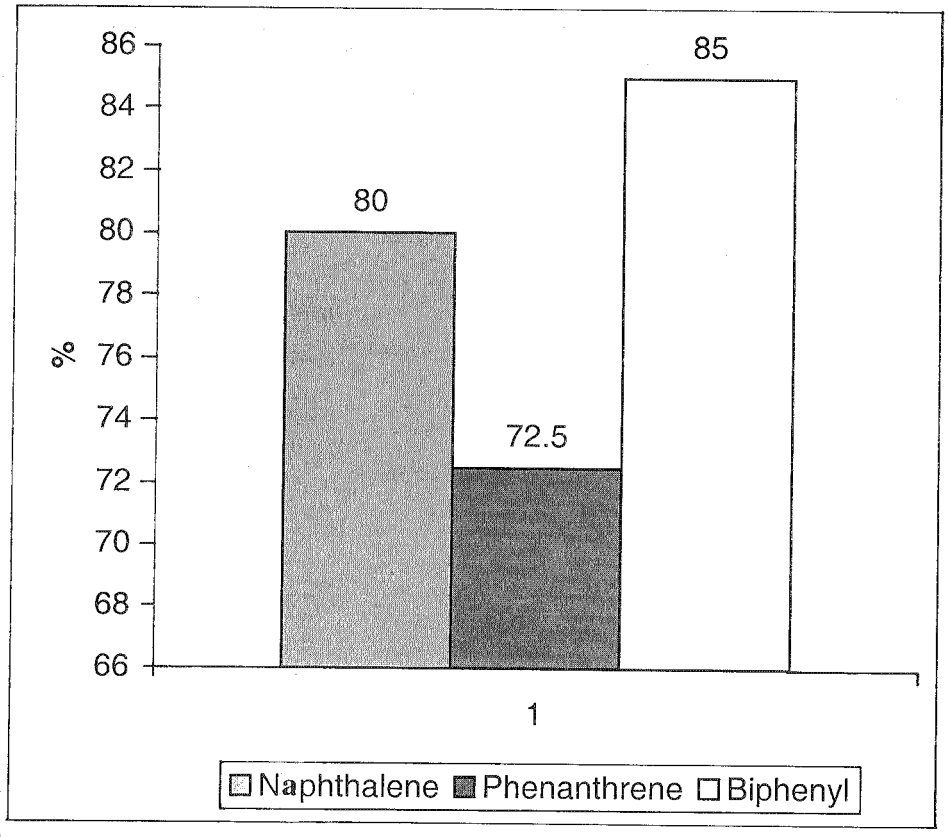

Fig. 1. Percentage of marine isolates showing the degradation of solid hydrocarbons.

Table 2. Acridine orange mediated curing of hydrocarbon degradative plasmids of marine isolates.

Hydrocarbon source	Total no. of strains used	Cured isolates					
		Cumulative results					
		Cured	%	Complete cured	%	Partial cured	%
Naphthalene	10	6	60	4	67	2	33
Octane	11	9	82	8	89	1	11
Xylene	5	4	80	4	100	0	0

Table 3. ***In vivo*** **gene transfer between naphthalene degrading (D) and the recipient(Re) non-naphthalene degrading marine isolates.**

Culture	Strain	Markers or characteristics		
		Antibiotics		Hydrocarbon
		Gentamicin (Gm) (250µg/mL)	Ampicillin (Amp) (250µg/mL)	Naphthalene
Recipient				
	SI 30	R	S	-
	SI 05	S	R	-
	SI 11	R	S	-
Donor				
	SI 22	-	S	+
	SI 21	-	S	+
	SI 20	-	S	+
	SI 29	S	S	+
	SI 32	-	S	+
	SI 24	S	R	+
Trans-conjugants(TC)	**D/ R**			
	20/05	S	R	+
	21/05	S	R	+
	22/05	S	R	+
	24/30	R	R	+
	29/11	R	S	+
	32/05	S	R	+

Keys: R= Resistant, Re= Recipient, S= Sensitive, TC= Trans-conjugant, D= Donor
+ = Hydrocarbon degrading strains.
- = Non degrading strains.

Table 4a. ***In vivo*** **retrotransfer of naphthalene degrading plasmid markers from transconjugants (D) to the cured (Re) marine isolates.**

Culture	Strains	Hydrocarbon marker (naphthalene)
Recipient (Cured)	SI 15	-
	SI 22	-
	SI 06	-
	SI 07	-
	SI 33	-
	SI 20	-
Donor (Transconjugants)	SI Tca (29/11)	+
	SI Tcb (30/24)	+
Transconjugants	TC_1 (TCa/ 15)	+
	TC_2 (TCa/ 22)	+
	TC_3 (TCa/ 06)	+
	TC_4 (TCa/ 07)	+
	TC_5 (TCa/ 33)	+
	TC_6 (TCa/ 20)	+

Keys: + = Hydrocarbon degrading strains.
- = Non-degrading strains.

Table 4(b). *In vivo* retrotransfer of octane degrading plasmid marker from transconjugants (D) to the cured(Re) marine isolates.

Culture	Strains	Marker Hydrocarbon (Octane)
Recipient (Cured)	SI 28	-
	SI 31	-
	SI 32	-
	SI 22	-
	SI 21	-
	SI 33	-
Donor (Transconjugants)	TCa	+
	TCb	+
Transconjugants	TC_1 (TCb/28)	+
	TC_2 (TCa/31)	+
	TC_3 (TCb/32)	+
	TC_4 (TCa/22)	+
	TC_5 (TCa/21)	-
	TC_6 (TCa/33)	+

Keys: + = Hydrocarbon degrading strains.
- = Non-degrading strains.

Discussion

Results of the screening of marine bacterial isolates to degrade liquid and solid hydrocarbons and genetic manipulations (including the retrotransfer) have been reported in this manuscript. Marine microorganisms possess a marked ability to utilize, oxidize and degrade these pollutants (Atlas & Bartha, 1981). Accordingly, marine isolates, which produced clear zones or had grown on mineral salt medium (MM2) agar plates (In case of solid hydrocarbons) without glucose were considered to have degradative potential. Among solid hydrocarbons, biphenyl has been degraded by most of the isolates. These findings are in agreement with those of Hutzinger (1994) and Kiyohara *et al.*, (1983). All the solid sources were degraded by 63% of the marine isolates. About 8% of the marine isolates did not degrade any of these solid hydrocarbons.

Liquid hydrocarbons (benzene, heptane, n-octane, toluene and xylene) were also screened for microbial degradation. A total of 13% of the isolates could utilize (degrade) all the five hydrocarbons. However, none of these sources was degraded by 8% of the marine isolates. Only 10% of the isolates were able to degrade both liquid and solid hydrocarbons used in this study. The aerobic microbial degradation of n-alkanes (such as n-octane and n-heptane) has also been reported by Schippers & Schippers (2000). Grabic (1987) and Bell *et al.*, (1994) have documented the benzene metabolism by marine bacteria; while Yang & Shim (1994) have reported aerobic and anaerobic microbial degradation of toluene, xylene and benzene. Infact, marine bacteria require different range of optimum temperature for degrading the solid and liquid hydrocarbons (Beastall, 1977; Bell *et al.*, 1994).

Acridine orange mediated curing experiments revealed that hydrocarbon degradation gene(s) are plasmid-borne. Grund & Eaton (1996) reported the presence of NAH plasmid

for naphthalene degradation in surface water organisms. Similarly, TOL plasmid for toluene and xylene degradation was reported by Williams (1974). However, Kim *et al.*, (1996) have reported the chromosomal location of genes responsible for naphthalene degradation. Most of these degradative plasmids code for at least 10 enzymes that are involved in the catabolism of a particular substrate (Chakrabarty, 1983). Most of the cured isolates had lost their potential *in toto* to degrade xylene and octane while majority of the cured isolates were only partially cured to degrade naphthalene. Infact, a complete or partial curing is the function of the copy number of plasmids plus the scattered distribution of these copies (the ones residing away from the periphery are not affected by the curing agents).

Hydrocarbon degradative and antibiotic resistance markers were used to determine whether the plasmids are conjugative/ transferable or not. Transconjugants were counter selected on gentamicin and ampicillin supplemented media and screened for naphthalene degradation. Herrick & Madson (1997) had also reported the horizontal transfer of naphthalene degradation genes. Still in another regimen of mating experiments, the cured isolates were used as the recipient and the transconjugants (obtained in the earlier *in vivo* direct gene transfers) were applied as the donor (for retrotransfer matings). Interestingly, the transconjugants obtained thus, did show the biodegradation of hydrocarbons like naphthalene and octane. These findings are in agreement with the findings of Mergeny (1987). It would suggest that genetic biodiversity is of common occurrence in the marine bacterial populations. Retrotransfer of genes (with particular reference to the hydrocarbon degradation) is a positively attributed phenomenon.

Acknowledgement

Research grant received from the University of Karachi is gratefully acknowledged.

References

Ashraf, M. and M. Jaffer. 1992. Pollution assessment through trace metal analysis of sediments of four tributary rivers of Pakistan, draining into Arabian Sea. *Ind. J. Mar. Sci.*, 21: 292-295.

Atlas, R.M. and Bartha. 1981. Microbial degradation of petroleum hydrocarbons: An environmental perspective. *Microbiol. Rev.*, 45: 180-209.

Bell, C.E., P.T. Kostecki and E.J. Calabrese. 1994. In: Hydrocarbon contaminated soils and ground water (Ed.): E.K. Kostecki. Lewis Publishers, New York.

Beastall, S. 1977. Treatment of natural fat oil spillages. In: *Treatment of industrial effluents*. (Eds.): A.G. Calley, C.F. Foster and D.A. Stafford. Hodder and Stoughton, London. pp. 328-335.

Chakrabarty, A.M. D.A. Friella and L. Depp. 1978. Transposition of plasmid DNA segments specifying hydrocarbon and their expression in various microorganisms. *Proc. Natl. Acad. Sci.*, USA, 75: 3109-3112.

Chakrabarty, A.M. 1983. Molecular cloning and selection of degradative genes in bacteria. Symposia Sessions, XV- Int. Cong. Genetics, New Delhi. Pp. 78-79.

Frantz, B. and A.M. Chakrabarty. 1986. Degradative plasmids in *Pseudomonas*. In: The bacteria-vol. 10 (Ed.): J.R. Sokaleh. Academic Press, New York pp. 295-323.

Finnerty, W.R. and H. Attaway. 1992. Microbial desulfurization and denitrogenation of hydrocarbon. In: *Microbial Enhanced Recovery*. (Eds.): J.E. Zajic, O.C. Cooper, T.R. Jack and N. Kosarie, Penn Well Books, UK, pp.83-91.

Furukawa, J., J.R. Simon and A.M. Chakrabarty. 1983. Common induction and regulation of biphenyl, xylene, toluene and salicylate catabolism in *Ps. paucimobilis*. *J. Bacteriol.*, 154:1356-1362.

Grabic, G.D. 1987. Fate of microbial metabolites of hydrocarbons in coastal plains. *Aquifer. Environ. Sci. Technol.*, 29: 458-469.

Grund, E. and R.W. Eaton. 1996. Field experiment in situ naphthalene degradation. *Environ. Sci. and Technol.*, 30: 2099-2103.

Herrick, J.B. and E.L. Madson. 1997. Natural horizontal transfer of a naphthalene dioxygen gene between bacteria native to a coal tar contaminated field site. *Appl. Environ. Microbiol.* 63: 2330-2337.

Hirota, Y. 1960. The effect of acridine dyes on mating type factors in *E. coli. Proc. Natl. Acad. Sci. USA.* 46: 57-64.

Hutzinger, O.H. 1994. The chemistry of chlorinated biphenyl. CRC Press Inc. Cleveland. pp. 237-245.

Kim, E., G.J. Zylstra and C.E. Cernigna. 1996. Evidence for role of 2-chromune. *Lett.*, 153: 479-489.

Kiyohara, H., K. Nagoo and K. Yana. 1982. Rapid screen for bacteria degrading water insoluble solid hydrocarbons on agar plates. *Appl. Environ. Microbiol.*, 43: 454-457.

Kiyohara, H., M. Sugiyama, F.I. Mandello and D.T. Gibson. 1983. Plasmid involvement in the degradation of polycyclic aromatic hydrocarbons by a *Beijerineka* sp. BBRC III: 939-945.

Miller, JH. 1972. Experiments in Molecular Genetics (Cold Spring Harbor Lab., New York.

Mergeny, M. 1987. Shuttle transfer (heterotransfer of chromosomal markers mediated by plasmid PU LB 113. *Mol. Gen. Genet.*, 209: 61-70.

Qdum, E.P. 1971. Fundamentals of Ecology.3rd ed. (W. B. Saunders Co; Philadelphia) pp. 574-575.

Ripp, S. 2000. Controlled field release of oil bioluminescent by genetically engineered microorganisms for bioremediation monitoring process and control. *Environ. Sci. and Technol.*, 34: 846-853.

Schippers, K. and T. Schippers. 2000. Enhanced degradation of phenenthrene in presence of surfactants. *J. Bacteriol.*, 83: 189-198.

Sutherland, J.B. and C.E. Cerniglia. 1995. Mechanism of polycyclic aromatic hydrocarbons degradation of toxic organic chemicals. (Eds.): L.Y. Yang and C.E. Cerniglia. Wiley, New York, pp. 269-306.

Williams, P.A and K. Murray. 1974. Metabolism of benzoate and methyl benzene by *Ps. putida.* Evidence for the existence of a TOL plasmid. *J. Bacteriol.*, 127: 1217-1224.

Yang, S.T. and H. Shim. 1999. Biodegradation of benzene, ethyl benzene and o-xylene by a co-cultureof *Ps. putida* and *Ps. fluorescens* immobilized in fibrous bed bioreactor. *J. Bacteriol.*, 67: 99-112.

(Received for publication 20 September 2002)