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72

PLASMID-ASSOCIATED PHENANTHRENE DEGRADATION BY CHESAPEAKE BAY SEDIMENT BACTERIA

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ABSTRACT - A differential, phenanthrene-enrichment agar plating technique was used to isolate phenanthrene-degrading bacteria from phenanthrene-enriched Chesapeake Bay sediment. One of the isolates, a yellow pigmented, slime-producing, Gram- negative rod identified as *Flavobacterium* sp. has been studied in detail. It has been found to carry a single plasmid with a mass of about 34 megadaltons. Results of hydrocarbon adherence tests showed that the organism adhered only minimally to n-octane and n-hexadecane, but emulsified cyclohexylbenzene and 1, 2, 3, 4, -tetrahydronaphthalene (tetralin). Curing of the plasmid with 3 $\mu g/ml$ novobiocin resulted in loss of phenanthrene clearing ability. Ability to degrade phenanthrene was confirmed using ^{14}C-labeled phenanthrene.

Key words : phenanthrene, plasmid, degradation, differential, enrichment, adherence, emulsification.

RÉSUMÉ - Une technique distinctive sur boîtes d'agar enrichies en phénanthrène a été utilisée pour isoler des bactéries dégradant le phénanthrène à partir du sédiment enrichi en phénanthrène de la baie de Chesapeake. Un des isolats, un bâtonnet pigmenté en jaune, produisant du mucus, Gram négatif et identifié comme étant un *Flavobacterium* sp a fait l'objet d'une étude détaillée. Il est porteur d'un seul plasmide, d'une masse de 34 mégadaltons. Les résultats des tests d'adhésion aux hydrocarbures montrent qu'il se fixe faiblement seulement au n- octane et au n-hexadécane, mais émulsionne le cyclobenzène et le 1, 2, 3, 4, -tétrahydronaphtalène (tetralin). Un traitement du plasmide par 3 ug/ml de novobiocine aboutit à la perte de sa capacité à dégrader le phénanthrène. La capacité à dégrader le phénanthrène est confirmée par utilisation de phénanthrène marqué au ^{14}C.

Mots clés : phénanthrène, plasmide, dégradation, distinctif, enrichissement, adhésion, émulsification.

INTRODUCTION

For the past several decades, chronic oil pollution in the marine environment with its attendant tainting of seafood, and entry of potentially carcinogenic components of the aromatic fraction of crude oil into the food chain, was a problem that has directed the attention of researchers towards hydrocarbon biodegradation. Also, the proposed commercial production of synthetic fuels from coal as a means of alleviating shortages of liquid hydrocarbon fuels, strengthens fears about environmental hydrocarbon pollution and concomitant dangers posed to human health. Polycyclic aromatic hydrocarbons (PAHs) are widely distributed in the environment and they are formed partly as a result of high temperature pyrolytic processes (Blumer, 1976). They are important contaminants because they persist in natural ecosystems (Platt and Mackie, 1980). These compounds find their way into the estuarine and marine ecosystems through natural submarine seepage of petroleum and accidental spillage which occur in the production, transport, and utilization of petroleum. Additionally, low amounts of PAHs are released into the environment by a wide variety of natural and man-made sources, such as forest fires,

incomplete combustion of organic material, motor vehicle emissions, coal liquefaction and gasification processes, cigarette smoke, and industrial wastes (Blumer, 1976).

Using a replica plate method, it has been observed that hydrocarbon-polluted sediments in central and northern Chesapeake Bay are enriched in phenanthrene-degrading microorganisms (Cooney and Shiaris, 1982, West *et al.,* 1984). Phenanthrene is not acutely toxic, carcinogenic, or mutagenic ; however, it has been used as a model substrate in studies of environmental degradation of polycyclic aromatic hydrocarbons, since its structure is found in the nucleus of carcinogenic PAHs, such as benzo(a) anthracene and 3-methylcholanthrene (Cerniglia and Yang, 1984). Hydrocarbon degrading bacteria play an important role in the clean-up of oil pollution (Colwell and Walker, 1977 ; Atlas, 1981) and in the cycling of carbon in the environment (Gibson, 1977). Plasmid mediation of key steps in the degradation of hydrocarbon molecules is now well established (Chakrabarty, 1980 ; Yen and Gunsalus, 1982). In general, degradation requires an interaction between chromosomal and plasmid genes (Fennewald *et al.,* 1978 ; Zuniga *et al.,* 1981).

It has been suggested that phenanthrene degradation may be under plasmid control in *Alcaligenes faecalis* (Kiyohara *et al.,* 1982). Although they did not definitively associate plasmids with oil degradation, Hada and Sizemore (1981) found that plasmid incidence was greater in *Vibrio* spp. isolated from an oil field in the northwestern Gulf of Mexico than in isolates from pristine control sites. It was, therefore, the aim of our study to examine the plasmids in phenanthrene-degrading estuarine bacteria for their ability to mediate the catabolism of this recalcitrant environmental pollutant.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used in this study are listed in Table 1. Details of the methods employed in isolating and identifying *Vibrio* sp. W10, *V. fluvialis* W20 and *Klebsiella/Enterobacter/Hafnia/Serratia* group strains W32, W33, S53 and S54 have been reported elsewhere (West *et al.,* 1984). *Flavobacterium* sp. strains SB23 and W45, as well as the unidentified Gram negative rods, were isolated on estuarine salts agar (ESWA) plates (Sherga *et al.,* 1979). The surface of each plate was uniformly spread with 0.2 ml of a filtered ethanol solution of phenanthrene (containing 0.5 g phenanthrene per 100 ml), using the method of Shiaris and Cooney (1983). All isolates were maintained on ESWA slants onto which crystals of phenanthrene were sprinkled.

Preparation of Deoxyribonucleic Acid (DNA)

One and one-half milliliters of late logarithmic phase cultures grown in Luria broth (casein Hydrolysate, 10 g ; NaCl, 10 g ; yeast extract, 5 g ; deionized water, 1 liter) at 30°C were introduced into 1.5 ml microcentrifuge tubes and centrifuged in a microcentrifuge (Eppendorf model 5412, Brinkman Instruments, Inc., Westbury, N.Y.). The cell pellet was lysed and DNA preparation obtained using the procedure described by Kado and Liu (1981).

Agarose gel electrophoresis

The agarose gel electrophoresis technique described by Meyers *et al.* (1976) was used for bacterial plasmid screening. Following electrophoresis, plasmids were detected by staining the gel slab in ethidium bromide (Sigma), 0.5 μg/ml, for 2 hr at room temperature. Photographic records were prepared using a Polaroid MP4 land camera.

Strain	Plasmid molecular weights (Mdal)
Unidentified Gram-negative rod W1	80, 57, 50, 38, 4.3, 2.2, 1.7, 1.3, 1.0
Unidentified Gram-negative	75, 6.0, 2.9, 2.3, 1.2
Unidentified Gram-negative rod W5	68, 57, 50, 4.3, 2.2, 1.7, 1.3, 1.0
Unidentified Gram-negative rod W6	80, 5.0, 1.9, 1.4, 1.1
Vibrio sp. W7	57
Unidentified Gram-negative rod W8	44, 31, 2.9, 1.3, 1.1, 1.0
Vibrio sp. W10	108, 70, 57, 40, 4.0, 1.6, 1.3, 1.1, 1.0
Unidentified Gram-negative rod W11	108, 74, 68, 50, 9.5, 5.6, 2.3, 1.5, 1.2
Unidentified Gram-negative rod W14	31,28
Vibrio fluvialis W20 (pG01, pG02)	36. 2.1
Unidentified Gram-negative rod S28	105, 77, 68, 60, 3.4, 3.3, 3.1
Unidentified Gram-negative rod W29	77
Klebsiella/Enterobacter/Hafnia/Serratia W	32 44, 2.3
Klebsiella/Enterobacter/Hafnia/Serratia W	33 98, 68, 60, 6.0, 5.6
Unidentified Gram-negative rod W42	92
Flavobacterium sp. S45	60
Unidentified Gram-negative rod S51	44, 38, 36, 2.5, 1.9
Klebsiella/Enterobacter/Hafnia/Serratia S53 (pG03, pG04)	34.3, 2.9
Klebsiella/Enterobacter/Hafnia/Serratia S54	96, 2.0
Flavobacterium sp. SB23 (pG05)	34
Flavobacterium sp. SB23 cured strain	-

Table I : Plasmids harbored by phenanthrene-degrading bacteria isolated from the Chesapeake bay.

Determination of the molecular weights of the plasmids.

Bacterial strains carrying reference plasmids of known molecular weights (mw) were obtained through the courtesy of Dr. E. Lederberg, Plasmid Reference Center, Stanford University, Palo Alto, Calif. The plasmids were TP116 (143.7 megadaltons, Mdal), RP4 (34 Mdal), R678 (62 Mdal), JR67 (57.2 Mdal). Also used were the plasmids carried by *Escherichia coli* strain V517 (Macrina *et al.*, 1978) received from Dr. D. Kopecko (Walter Reed Army Institute of Research, Washington, D.C.).

Curing experiments

All the curing treatments were performed with organisms grown in L broth using the curing protocol of Salisbury *et al.* (1972) with some modifications. Cultures containing the highest concentration of curing agent in which growth was clearly visible were inoculated into fresh L broth tubes containing the same concentration of curing agent and incubated for another 24 hr. Tubes showing visible growth were then diluted and spread onto tryptic soy agar (TSA) plates. Colonies from the TSA plates were replicated with velvet pads to ESWA plates overlaid with phenanthrene. Colonies showing no detectable clearing of phenanthrene were picked up from the master plates and screened for loss of plasmids.

Transformation experiments

A modification of the transformation procedure of Mandel and Higa (1970) was used. An overnight Upper Bay Yeast Extract (UBYE) broth culture was diluted 1:100 into fresh UBYE broth at 30°C. The medium contained yeast extract, 1.0 g; protease peptone, 1.0 g; NaCl, 23.4 g; KCl, 0.75 g; $MgSO_4 . 7H_2O$, 6.98 g; deionized water, 1 liter; pH 7.4. The cells were collected at A_{500} nm = 0.6 and sedimented at 5 000 rpm for 5 mn at 2°C (Sorvall RC-2B, Dupont Instruments, Newtown, Conn.) Following resuspension in 20 ml of 50 mM $CaCl_2$ for 30 min at 0°C, one tenth milliliter of cells was added to plasmid DNA

(isolated by electroelution from 0.7 % agarose gels as described by Maniatis *et al.*, 1982) contained in 0.1 M Tris buffer, pH 7.2, and held for 10 min at 0°C. The cell suspension was subjected to the following heat treatment : incubation for 10 min at room temperature, followed by 2 min at 37°C plus 10 min at room temperature. One milliliter of UBYE broth was added to the cells and the suspension incubated at 37°C for 20 min. Following this incubation, 2.5 ml of ESW soft agar at 47°C was added to each tube of cell suspension. The contents were mixed and poured onto ESWA plates overlaid with phenanthrene.

Radiolabeled (^{14} C) -phenanthrene metabolic experiments

Five microliters of (9 - ^{14}C) -phenanthrene (Amersham Corp., Arlington Heights, Illinois) in hexane were added to 1/4 inch diameter dry sterile blank paper discs (BBL Microbiology Systems, Cockeysville, MD) in a glass petri dish, yielding a nominal concentration of 41 ppb of phenanthrene and a count of 11,000 dpm. The paper discs were allowed to stand for 10 min for the hexane to evaporate. One ^{14}C -phenanthrene-impregnated disc was introduced into a Durham tube. To the Durham tube containing the disc was added 0.3 ml of minimal salts broth. Each Durham tube was introduced into a larger vial containing 2 ml of preblend 3a80 scintillation cocktail/carbon dioxide absorbent (Research Products International, Elk Grove Village, Illinois) to drap the $^{14}CO_2$ evolved. To each Durham tube was added 0.1 ml of culture. Two replicate batches of vials similarly prepared were capped and incubated at 25°C. The two triplicate batches of vials in the experiment included : 1) uninoculated controls, 2) *E. coli* HB101, 3) *Flavobacterium sp.* SB23 (pG05), and 4) cured derivative of strain SB23. At the end of the incubation period, the discs were removed. One batch of three replicates was counted after 2 days and the other batch after 7 days, using the Beckman LS 7500 microprocessor-controlled liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Percent of total above uninoculated control was determined using 11,000 counts as initial disintegrations per minute (dpm) per vial, i.e,

$$\% \text{ of total above control} = \frac{\text{dpm}_X - \text{dpm}_C}{11{,}000} \times 100$$

where the subscrips, x = test vial, and c = uninoculated control.

Hydrocarbon adherence/emulsification tests

Adherence to, and emulsification of, selected liquid hydrocarbons was investigated using the method of Rosenberg and Rosenberg (1981) with some modifications. The hydrocarbons tested were n-octane, n-hexadecane, cyclohexylbenzene, and 1, 2, 3, 4, -tetrahydronaphthalene (tetralin) all of which were obtained from Chemical Samples Co., Columbus, Ohio. Cells harvested at stationary phase (48 hr) from 50 ml of UBYE broth culture grown at 30°C with shaking, at 120 rev/min were washed twice with PUM buffer (K_2HOP_4 . $3H_2O$, 22.2 g ; KH_2PO_4, 7.26 g ; urea, 1.8 g ; $MgSO_4$. $7H_2O$, 0,2 g ; deionized water, 1 liter ; pH, 7.1). Washed cells were then resuspended in PUM buffer to an absorbance of 1.5 at 400 nm (Gilford spectrophotometer model 2600, Corning Glass Works, Overlin, Ohio). To round bottom acid-washed test tubes (10 mm diameter) containing 1.5 ml of washed cell suspension were added various volumes (0.1, 0.2, 0.3, and 0.4 ml) of the test hydrocarbons. Other steps in the procedure were according to Rosenberg and Rosenberg (1981).

RESULTS AND DISCUSSION

Plasmids in phenanthrene-degrading bacteria from Chesapeake Bay varied greatly in molecular weight (Table 1) and were randomly distributed between 1 and 110 Mdal. The number of plasmids harbored by each strain differed substantially. Some strains possessed as many as nine distinct plasmids, e.g., strains W1, W10, and W11, while others harbored but a single plasmid, i.e., strains W7, W29, W42, W45 and SB23. There was a higher incidence of plasmid carriage in strains isolated from the water (W) column (4.6 plasmids per plasmid-bearing strain) than in sediment isolates (3.0 plasmids per plasmid-bearing strain). Only 30 % of the plasmid-containing strains were sediment isolates. The size distributions of plasmids carried by phenanthrene degrading bacteria in water and sediment samples also differed. Water column isolates tended to have small plasmids. More than one half (54.3 %) of the plasmids detected in water column isolates fell within the 1-10 Mdal size range, compared to 38.5 % of plasmids for sediment strains. Conversely, sediment isolates had large plasmids. Almost half (46.1 %) of the plasmids borne by the sediment isolates were in the 31-50 Mdal mw class, compared to 12.9 % of plasmids in water column strains. Carriage of plasmids in other size ranges was low. No sediment bacteria possessed plasmid bands in the 11-30 Mdal mw range. Poor detection of plasmids in this size class may be due to the fact that chromosomal DNA fragments band in this area, thereby obscuring plasmid DNA bands.

Generally, the frequency of plasmid carriage was higher in strains obtained under selective conditions than in natural, randomly selected heterotrophic populations. The moderately high percentage (36 %) of plasmid carriage observed in our study was not unexpected, when compared to similar findings reported by other investigators. Hada and Sizemor (1981) have reported that 35 % of 440 *Vibrio* strains isolated from an oil field in the northwestern Gulf of Mexico, without selection, carried plasmids, compared to 23 % of *Vibrio* strains from non-oil- impacted control sites.

In a closely related study in our laboratory of plasmid incidence in bacteria isolated from polluted and non-polluted Atlantic Ocean samples, it was found that bacteria associated with toxic chemical waste more frequently contained plasmid DNA and demonstrated antibiotic resistance than bacteria isolated from domestic sewage-impacted waters or from uncontaminated open ocean sites (Baya *et al.*, 1986). In that study, it was observed that plasmid molecular weights ranged from 1.4 to 151 Mdal. Plasmids in the 2.5 - 6.3 Mdal mw were most common, while those between 12.6 and 20.0 Mdal mw were uncommon. While plasmids were detected in bacteria from all sites, the largest number of plasmids was found in sewage effluent strains. The number of plasmids possessed by each strain also varied, for the whole data set and between sites. Sewage effluent (SE) strains possessed an average of 2.6 plasmids per strain, as opposed to 1.2 plasmids per strain for sewage outfall (SO). The Ocean City (OC) and clean water (CW) strains each showed an average of less than one plasmid for every two strains (Baya *et al.*, 1986).

Novobiocin proved effective in curing the single 34 Mdal plasmid in *Flavobacterium* sp. SB23 (Fig. 1). Loss of phenanthrene clearing ability by the cured derivative strain of SB 23 is concluded to be a consequence of plasmid elimination. Some investigators have suggested that the phenanthrene degrading phenotype of a soil isolate identified as *Alcaligenes faecalis* strain AFK2 may be under plasmid control. The phenanthrene-degrading (Phn_+) phenotype of this organism disappeared after twenty successive subcultures in a minimal salts medium containing o-phthalate or after subculture in nutrient broth containing mitomycin C (Kiyohara *et al.*, 1982). However, no actual screening for plasmids was reported by these workers. We report here the first physical evidence of plasmid loss correlated with loss of phenanthrene-degrading phenotype in *Flavobacterium* sp. SB23.

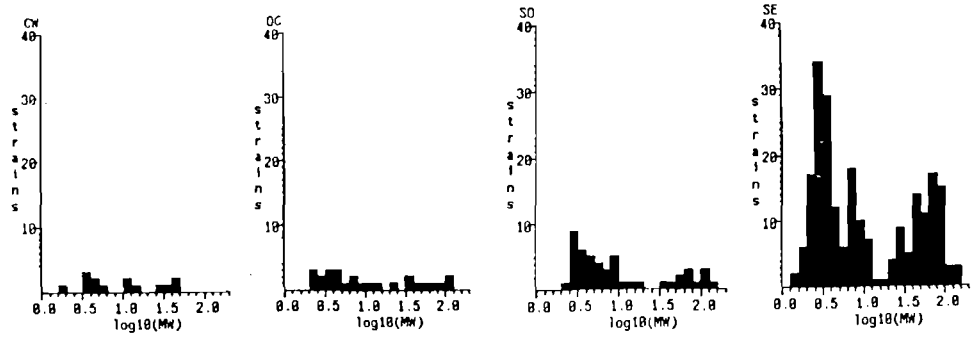

Figure 1 : Frequency of plasmid molecular weights in sewage effluent (SE), sewage outfall (SO), Ocean City (OC), and clean water (CW) strains.

The results of the experiment designed to transfer the 34 Mdal plasmid back into the cured derivative strain of SB23 were inconclusive. Plasmid DNA could not be detected in the transformed cells, even though clearing was observed around the colonies two to four days after cells undergoing transformation treatment were spread-inoculated onto phenanthrene-layered agar plates. Lack of detection of the plasmid DNA suggests that integration of the plasmid into the chromosome may have occurred, as one possible explanation for this finding.

Strains	After 2 days		After 7 days	
	DPM*	% of total DPM above control	DPM	% of Total DPM above control
Control	223	0.00	718	0.00
E. coli HB101	290.83	0.62 = 0.01	693.93	-0.22 = 0.01
Flavobacterium sp. SB23 cured	286.33	0.58 = 0.05	581.5	-1.24 = 1.43
Flavobacterium sp. SB23 (pG05) parent	391.67	1.50 = 0.1	1295.7	5.25 = 0.13

Table 2 : Radiolabeled (^{14}C-) Penanthrene Metabolism. * mean DPM (disintegrations per minute) for three replicate vials.

Flavobacterium sp. SB23 (pG05), known to clear phenanthrene agar, and cured dervivative (non-phenanthrene clearing) strain were tested for their abilities to metabolize (9 -^{14}C) - phenanthrene as sole source of carbon and energy (Table 2). While *E. coli* HB101, a strain not known to degrade phenanthrene, yielded dpm of 0.62, 0.01 % above control after 2 days of incubation, the cured derivative of strain SB23 yielded dpm of 0.58, 0.05 % above control. The parent *Flavobacterium* sp. SB23 carrying the 34 Mdal plasmid, pG05, yielded dpm of 1.5, 0.11 % above control after 2 days of incubation. After 7 days of incubation, however, both *E. coli* HB101 and the cured strain of SB23 yielded counts which were below that of control. The parent *Flavobacterium* sp. SB23 (pG05) yielded dpm of 5.3, 0.13 % above control after 7 days of incubation. From these results, it is concluded that the plasmid-bearing strain showed an increased $^{14}CO_2$ evolution from ^{14}C-phenanthrene. In one experimental sample, counts for the plasmid-bearing strain were significantly greater than those of the cured strain. These results suggest that

phenanthrene degradation by *Flavobacterium* sp. SB23 (pG05) is influenced by a plasmid-mediated metabolic pathway. The ^{14}C-phenanthrene results reconcile the phenanthrene clearing phenomenon observed on agar plates, used in isolation of presumptive phenanthrene-degrading bacteria in this study and reported as a rapid screening method for phenanthrene-degrading bacteria (Cooney and Shiaris, 1982 ; Shiaris and Cooney, 1983 ; Kiyoharaa *et al.*, 1982). In contrast, Fedorak *et al.* (1984) detected no significant release of $^{14}CO_2$ from cultures of yeasts and fungi to which had been added Prudhoe Bay crude oil "spiked" with (9-^{14}C)-phenanthrene. Even though the organisms used were known to attack n-alkanes in the crude oil, they failed to mineralize (1-^{14}C)-naphthalene and (9-^{14}C)-phenanthrene added to the crude oil (Fedorak *et al.*, 1984).

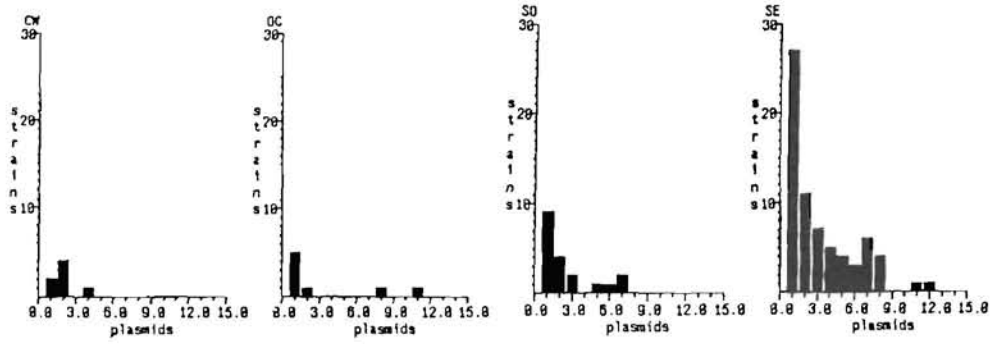

Figure 2 : Frequency of number of plasmids in sewage effluent (SE), sewage outfall (SO), Ocean City (OC), and clean water (CW) strains.

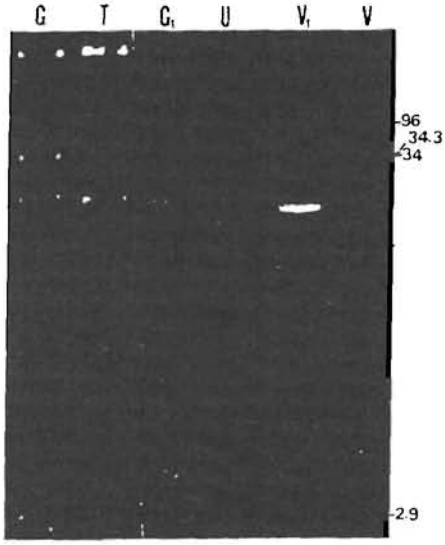

Figure 3 : Plasmid NA banding patterns of *Klebsiella/Enterobacter/Hafnia/Serratia* strain S53 (pG03, pG04), *Flavobacterium* sp. strain SB23 (pG05), and cured derivatives S53 (pG03) and SB23. Lane designations are : G= Parent strain S53 (pG03, pG04) ; T= chromosomal DNA ; G1 = cured derivative strain S53 (pG03) ; U = RC 709 (R40a) (reference strain) ; V_1 = cured derivative strain SB23 ; V = parent strain SB23 (pG05). Plasmid molecular weights corresponding to bands present in DNA preparations of the strains are indicated on the right.

Adherence and emulsification patterns of the phenanthrene- degrading *Flavobacterium* sp. SB23 bearing the 34 Mdal plasmid and the cured derivative strain are shown in Figure 2. The variable patterns of adherence or emulsification exhibited by both strains tested may be partly due to differences in extra- cellular polysaccharide production. For instance, while the slime- producing *Flavobacterium* sp. SB23 bearing the 34 Mdal plasmid adhered minimally to n-octane and n-hexadecane (aliphatic hydrocarbons) and strongly emulsified cyclohexyl-benzene and tetralin (aromatic hydrocarbons), its cured derivative strain showed minimal adherence to all four hydrocarbons tested. Our data suggest that the 34 Mdal plasmid may also be involved in either production or regulation of the biosynthesis of extracullular, emulsifying polysaccharides produced by strain SB23. Zajic *et al.* (1974) noted that the major emulsification process involved in a massive oil spill was attributed to a microbial emulsification. Since emulsification demonstrated in the present study occurred only when strains were incubated in the presence of aromatic hydrocarbons, and not straight chain aliphatics, hydrocarbon structure may be a determining factor in this process.

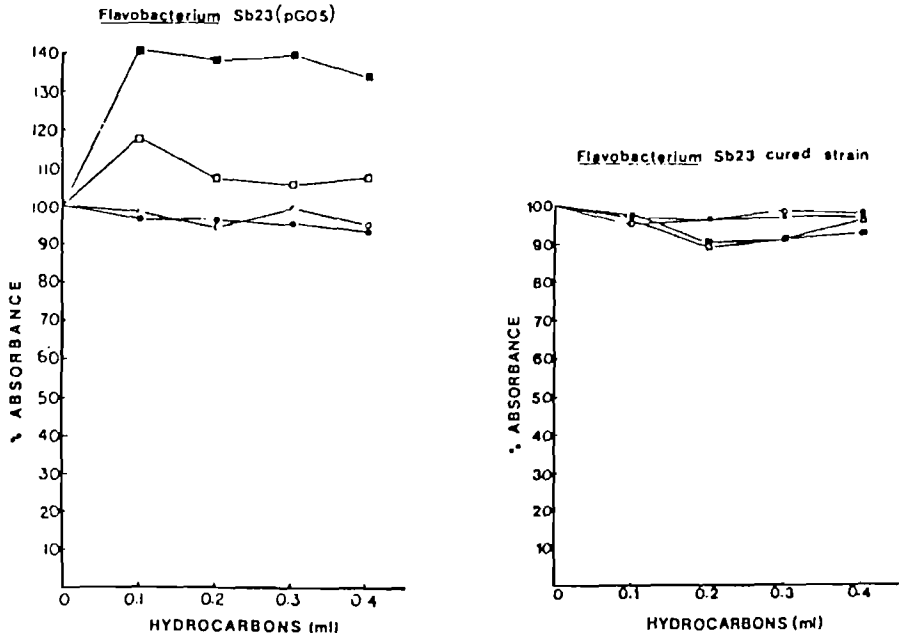

Figure 4 : Adherence of *Flavobacterium* sp. strain SB23 (pG05) and its cured derivative strain to liquid hydrocarbons; (○) n-octane, (●) n-hexadecane, (□) cyclohexyl-benzene, (■) tetralin.

In conclusion, many workers have shown that plasmids carrying genes influencing degradation of hydrocarbons abound in bacterial populations. Because these bacteria are widely distributed in chronically polluted estuarine environments, plasmid carriage may provide genetic control for adherence to, or emulsification of, hydrocarbons, ultimately resulting in removal via biodegradative pathway(s). In this study, plasmid DNA was found to be associated with clarification of phenanthrene-degrading *Flavobacterium* species.

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