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# Incidence of Plasmids in Marine Bacteria Isolated from the Bunker-C Oil Enriched Culture

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# Bunker-C유 집식배양으로부터 해양세균 Plasmid의 분포

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**Samples used for the enrichment culture were collected from the sea water of suspected chronic petroleum contamination in the vicinity of Pusan, Chungmu and Ulsan ports, Korea. Alkaline lysis and agarose gel electrophoresis techniques were employed to screen these isolates for the presence of plasmid DNA. There were a little differences in the percentage of isolates containing plasmids between sampling sites of unpolluted sea water (22%) and polluted sea water (25%). Bacterial isolates taken from the Bunker-C oil enriched culture showed significantly more plasmid incidence (29%). About two thirds of strains grown on a variety of hydrocarbons were Gram negative strains of which 33% contained one or more plasmids. Multiple plasmids were observed in 23% of the plasmid-carrying strains. Forty one percent of the plasmids detected were estimated to have a mass of 20 kb or more.**

There have been some examinations to determine the incidence of plasmids in natural populations of terrestrial bacteria (1-4), medically important bacteria (5,6), or plant pathogens (7), but few reports have dealt with the incidence of plasmids in natural populations of marine environments. Hada and Sizeman (8) examined the plasmid incidence of marine *Vibrio* species in the Gulf of Mexico and found the occurrence of plasmids to be higher in oil field regions (35%) than in the control area (23%). Glassman and McNicol (9) found that 46% of the estuarine bacteria from the sediment and water column in Chesapeake Bay carried plasmids. Simon *et al.* (10) isolated 58 marine luminous bacteria mainly from the Mediterranean and Red seas and found that 43% of the bacteria carried plasmids. The marine environment has been characterized as nutritionally starved (11). Therefore, the effects of an energy-limiting situation will negatively influence the maintenance of nonessential factors such as plasmids, since a balance between genetic flexibility and the metabolic load must be maintained (12, 13). In this work, bacterial strains were collected from locations within the suspected chronically polluted harbor and maintained in Bunker-C oil enriched culture artificially. Bunker-C oil is one of the most dominant sea pollutant in the vicinity of Korea. In the marine environment also, enrichment process may be an influence in the maintenance of plasmids in marine bacteria.

Then plasmid multiplicity could be used as an indicator of some types of water pollution. In addition, plasmids in marine bacteria must be studied to understand ecological implications of adaptation to an environmental situation such as the oil pollution. This work was done to understand the incidence of plasmid DNA among the clean site, polluted site, and Bunker-C oil enriched culture. The associations were also compared between hydrocarbon utilization and plasmid to study the adaptation or

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function of plasmids in marine bacteria.

## Materials and Methods

### Sampling sites

Sampling sites were chosen in areas thought to have been chronically polluted with oil, namely shipping ports of Pusan, Chungmu and Ulsan in Korea. Sea water sample of unpolluted site was obtained from Haeundae beach, Pusan, Korea

### Enrichment culture

Freshly collected sea water samples were inoculated in 500m*l* linger's bottle containing 20m*l* of Bunker-C oil sea water medium (Bunker-C oil, 7.8g; $K_2HPO_4$, 0.01g; $(NH_4)_2SO_4$, 1g; Tris-aminomethane, 12.1g; sea water, 1 liter; pH 7.6). Bunker-C oil was obtained from Yukong petroleum refinery as a 4.0% of high sulfur content. It was incubated on rotary shaker of 12rpm at 18 °C. Following the appearance of turbid growth within 5 days, 600u*l* of culture was transferred to a second bottle containing the oil medium and incubated for 5 days more. This procedural cycle of incubation was repeated 5 times in total. The final enrichment culture was obtained from the 5th transfer.

### Bacterial isolation

Each sea water or culture sample was diluted with sterile sea water and plated on modified marine agar 2216E (14); Bacto-peptone, 5g; Yeast extract, 1g; Ferric citrate, 0.1g; $NH_4NO_3$, 0.0016g; $Na_2HPO_4$, 0.008g; sea water, 1 liter; pH 7.6. Isolates were picked randomly after 7 days of incubation at 18 °C and transferred to the modified marine broth 2216E to extract plasmid DNA.

### Growth on sole hydrocarbon

Bacteria from the enriched culture were tested for their ability to grow on 0.05 % of hydrocarbon compounds, such as camphor, naphthalene, phenol, toluene and xylene in the sea water medium. The colonies were grown up to 2 weeks at 18 C and tested for the presence of plasmid DNA.

### Cell lysis and plasmid isolation

Oil degrading bacteria were grown to early stationary phase in 3m*l* of the modified marine broth. Then the bacterial suspension was harvested and lysed with alkaline sodium dodecyl sulfate using the rapid isolation technique of plasmid DNA (15).

### Agarose gel electrophoresis

The lysate was subjected to 0.6% agarose gel electrophoresis for the detection of plasmid DNA, and the plasmid was visualized by the technique described by Meyers *et al.*(16). The molecular weights of the plasmids were determined by comparing them with four standard plasmid markers including pUC 9 (2.71 kb), pBR 322 (4.36 kb), YEP 13 (10.7 kb) and pGU 66 (18.14 kb).

## Results and Discussion

### Plasmid contents from unpolluted and polluted sites

Sea water of suspected chronic oil contamination was collected for the Bunker-C oil enriched culture, and also used to examine the presence of plasmids. Bacterial isolates obtained from the unpolluted and the suspect polluted sea waters exhibited a little different frequency of plasmid incidence as shown in Table 1. We tested 32 strains of marine bacteria from unpolluted sea water and found that 7 carried plasmids (22%), whereas 167 strains from polluted sea water revealed that 39 carried plasmids (25%). Other researches for plasmids in natural populations of marine bacteria have reported the following frequencies: 23% in an unpolluted site and 35% in a polluted site of the Gulf of Mexico (8); 46% in Chesapeake Bay (8); 43% in marine luminous bacteria from the Mediterranean and Red Seas (10); 28% in Antarctica (17). We believe that the actual frequency of plasmid occurrence may be higher than our estimated values since plasmid screening techniques are capable of detec-

**Table 1. Frequency of plasmid-carrying strains isolated from sea water of different sampling sites**

| Sites | No. of bacteria tested | No. of plasmid-carrying strains | % of strains with plasmids |
|---|---|---|---|
| Haeundae beach | 32 | 7 | 22 |
| Ulsan Port | 51 | 13 | 25 |
| Chungmu Port | 59 | 14 | 24 |
| Pusan Port | 57 | 12 | 26 |

ting plasmids only within a general size range and for a specific organism.

**Plasmid content from enrichment culture**

The plasmid content of the population from Bunker-C oil enriched culture (29%) as shown in Table 2 was greater than that of the population from inoculum (25%) or clean sea water (22%). We presume that this is an adaptation by the plasmid-carrying bacteria in response to the generally increased level of pollutants. Our estimated plasmid frequency is still below the values cited by other reports (8-10), but it is higher than the 23% value for the oil degrading bacteria isolated from the Gulf of Mexico (18).

**Table 2. Frequency of plasmid-carrying strains isolated from the Bunker-C oil enriched culture**

| | No. of bacteria tested | No. of plasmid-carrying strains | % of strains with plasmids |
|---|---|---|---|
| Inoculum | 167 | 39 | 25 |
| Enriched culture | 55 | 16 | 29 |

**Plasmid contents from sole hydrocarbon**

The use of hydrocarbon for bacterial cell growth was chosen to detect the relationship between the plasmid-mediated traits and oil biodegradation. The Bunker-C oil enriched culture was diluted and spread on each hydrocarbon-containing sea water medium. Approximately 50 colonies were selected randomly to test the relationships among the plasmid, Gram stain and shape as shown in Table 3. The result shows generally the high adaptation of plasmid-carrying bacteria, such as the plasmid incidence of average 35%, 38%, 31%, or 31% on naphthalene, phenol, toluene, or xylene, respectively. Therefore, it may be said that the plasmid-carrying bacteria are able to utilize the hydrocarbon more rapidly. Strikingly, dominant strains of Gram negative and rod bacteria on naphthalene or phenol possessed at least one plasmid, namely 83% or 67%, respectively. Twenty three percent of the plasmid-carrying strains showed multiple plasmid

**Table 3. Relationships among the plasmid-carrying bacteria, Gram stain and shape on sole hydrocarbon source**

| Hydrocarbon | No. of strains tested | Gram stain | Shape | No. of bacteria | No. of plasmid carrying strains | % of strains with plasmid |
|---|---|---|---|---|---|---|
| Camphor | 53 | + | rod | 11 | 1 | 9 |
| | | + | coccus | 9 | 2 | 22 |
| | | − | rod | 16 | 4 | 25 |
| | | − | coccus | 17 | 3 | 18 |
| Naphthalene | 51 | + | rod | 8 | 2 | 25 |
| | | + | coccus | 17 | 4 | 24 |
| | | − | rod | 6 | 5 | 83 |
| | | − | coccus | 20 | 7 | 35 |
| Phenol | 50 | + | rod | 6 | 2 | 33 |
| | | + | coccus | 14 | 5 | 36 |
| | | − | rod | 6 | 4 | 67 |
| | | − | coccus | 24 | 8 | 33 |
| Toluene | 51 | + | rod | 7 | 3 | 43 |
| | | + | coccus | 13 | 4 | 31 |
| | | − | rod | 17 | 3 | 18 |
| | | − | coccus | 13 | 6 | 46 |
| Xylene | 51 | + | rod | 7 | 3 | 43 |
| | | + | coccus | 6 | 3 | 50 |
| | | − | rod | 15 | 4 | 27 |
| | | − | coccus | 23 | 6 | 26 |

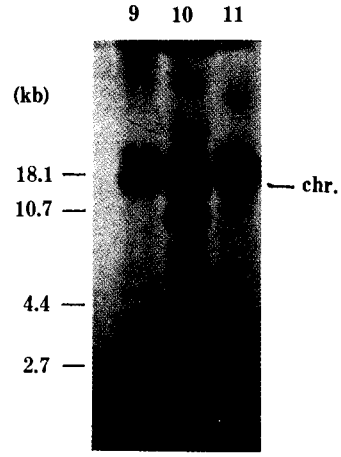


**Fig. 1. Identification of plasmids by agarose gel electrophoresis.**
Lane 1 to 3 are plasmids from naphthalene-utilizing bacteria NUB-9, 10 and 11, respectively.

bands ranging from 2 to 5 molecular weight species in an individual isolate. Five plasmid bands from a naphthalene-utilizing bacterium NUB-10 was the maximum number as shown in Fig. 1. Since one plasmid can give three bands of closed circular, open circular and linear DNAs, and the technique used in this work can detect replicative intermediate forms of plasmid as well as multimers, we are not certain how many separate plasmids these multiple bands represent.

**Molecular weight of plasmids**

Size classes of the plasmids isolated from Bunker-C oil enriched culture on the hydrocarbon sources such as camphor, naphthalene, phenol, toluene and xylene were estimated by comparing the relative migration with known plasmid markers. Forty one percent of the plasmids had a molecular weight larger than 20 kb namely 13 Mdal as shown in Table 4. Our result represents a preponderance of large plasmids in marine bacteria to be adapted to biodegrade chemical compounds in petroleum. This result generally agrees with that of Glassman and McNicol (9), who found a preponderance of small plasmids in marine isolates from clean sites but larger plasmids in isolates from a more polluted site.

**Table 4. Molecular weight of plasmids isolated from Bunker-C oil enriched culture**

| Plasmid size (kb) | No. of plasmids | Relative frequency (%) |
|---|---|---|
| above 20 | 72 | 41 |
| 10-20 | 45 | 25 |
| 5 -10 | 53 | 30 |
| below 5 | 7 | 4 |


## 요 약

우리나라 연근해역의 해양오염중 주종을 이루는 Bunker-C유를 대상으로 부산 및 충무, 울산항구의 해수를 균원으로 접종하여 enrichment culture시켰다. 이와같은 혼합배양 해양세균들의 plasmid DNA 분포를 agarose gel 전기영동상에서 조사하여 보았다. 우선 오염되지 않은 해수로부터 분리한 세균들의 plasmid 분포(22%)와 만성적으로 유류오염이 예상되는 각 항구의 세균 plasmid 분포(25%)에는 조금 차이는 있었으며 enrichment culture중에는 29% 정도로서 plasmid 분포비율이 증가하였다.

각종 탄화수소 화합물들에 성장하는 세균중 약 33%는 plasmid를 함유하고 있었으며, 또 약 62% 정도는 Gram 음성균이었다. Plasmid 함유 세균중 23% 정도는 2종 이상의 plasmid를 갖고 있으며, 41%의 plasmid는 20 kb 이상의 크기를 가지고 있는 것으로 나타났다.


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