油濁海域(備讃瀬戸)と非油濁海域(響灘)から分離した重油分解細菌の菌相および重油分解能

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Characterization and Oil-degrading Activity of Heavy Oil-degrading Bacteria Isolated from the Sea Water of Oil-polluted Bisan Seto and Oil-unpolluted Hibiki Nada*1

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The flora of heavy oil-degrading bacteria isolated from water samples collected in oil-polluted Bisan Seto and oil-unpolluted Hibiki Nada was investigated. The oil-degrading activity of the isolated strains was also measured. It was observed that coryneforms were exclusively dominant among oil-degrading isolates in oil-polluted Bisan Seto. In oil-unpolluted Hibiki Nada, coryneforms and *Pseudomonas* were isolated in the same ratio. Oil-degrading activity of coryneforms was much higher than that of *Pseudomonas*, regardless of where they were isolated. From these results, it was suggested that coryneforms play an important role in the biodegradation of oil in oil polluted Biasn Seto. When the average oil-degrading activity of the strains isolated from Bisan Seto and that from Hibiki Nada was compared, the former (21.6%) was significantly higher than the latter (14.1%).

It was suggested that the flora and oil-degrading activity of oil-degrading bacteria were sensitive indicators of oil-pollution than other factors previously examined (*i.e.* numbers of oil degrading bacteria, the ratio of the degraders to the total heterotrophs).

A large-scale oil spill accident so-called "Mizushima Oil Spill"[1] occurred in Seato Inland Sea on December, 1974. In our previous studies,[2-5] we compared the seasonal and locational distribution of heavy oil-degrading bacteria during 1975 in the sea water of oil-polluted Bisan Seto and that of oil-unpolluted Hibiki Nada (Yamaguchi Prefecture). However, we could not find a significant correlation between the number of heavy oil-degrading bacteria and the concentration of oil within each area. Therefore, a study of the characterization of oil-degrading microflra and oil-degrading activity of the oil-degrading bacteria in each area is necessary to asses the microbial capability of the areas to degrade oil pollutants.

In this study, we have attempted to characterize the flora of oil-degrading bacteria isolated from both oil-polluted Bisan Seato and oil unpolluted Hibiki Nada. We have also attempted to measure the oil-degrading activity of each bacterial strains isolated.

Materials and Methods

Characterization of Oil-degrading Microbial Flora

In 1975, water samples for bacterial enumeration and chemical analysis were collected on February, May, August, and November from Bisan Seto (Fig. 1) and on March, May, and September from Hibiki Nada (Fig. 2). The formar represented an oil-polluted area and the latter represented an oil-unpolluted area. At that time, 81 strains demonstrating utilization of oil as a sole carbon source were isolated from Bisan Seto, and 50 strains demonstrating such activity were isolated from Hibiki Nada following procedures already described.[6] These isolates have been mainteined since that time by monthly transfers into ZoBell 2216E agar slants supplied with 5 drops of desulfurized "Class C" fuel oil (the spilled fuel oil). In this study, these bacterial isolates were characterized according to the scheme of Shimidu[7] with additional information derived from Cowan and Steel.[8] All isolates were examined for morphology, spore formation, gram reacton, motility, catalase activity, oxidase activity, and the ability to oxidize or ferments

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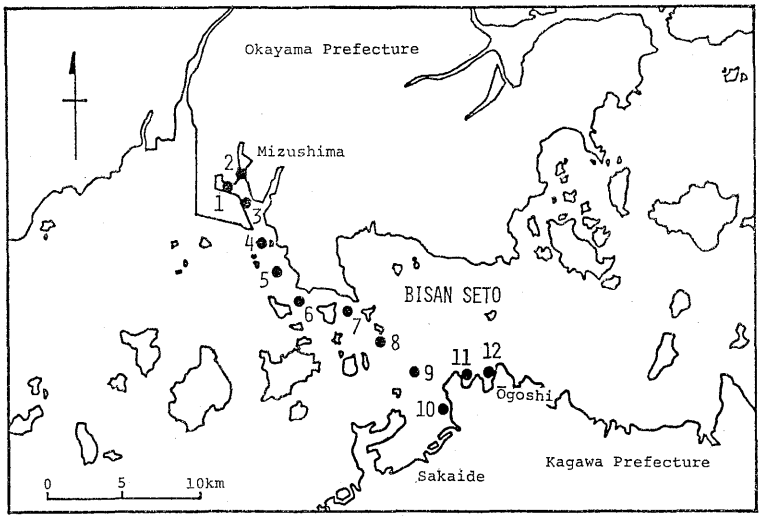

Fig. 1. Location of sampling stations in Bisan Seto (oil polluted waters).

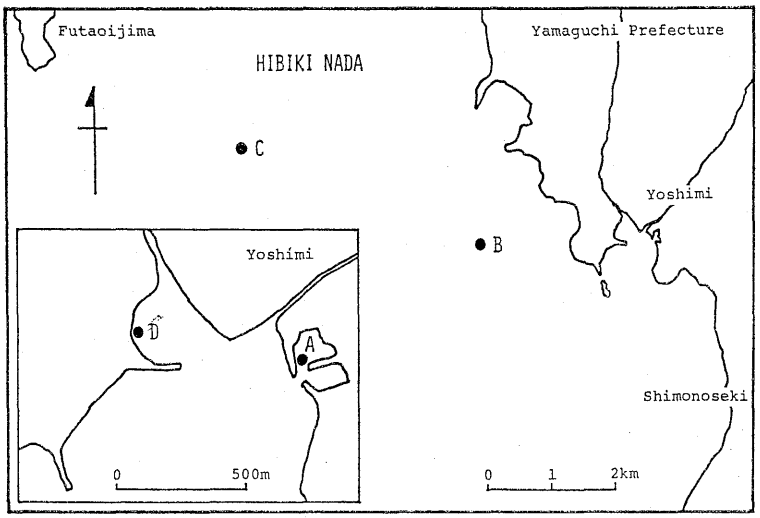

Fig. 2. Location of sampling stations in Hibiki Nada (control waters).

sugars. The ability of the isolates to oxidize or ferment sugars with and without acid or gas production was determined using a marine oxidation-fermentation (MOF) medium proposed by Leifson.[9]

Determination of Microbial Oil-degrading Activity

A basal salts medium was empolyed for the measurement of oil-degrading activity of isolated strains. This contained (g./*l.* distilled water, pH 7.5) NaCl, 30.0; KH_2PO_4, 0.5; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.2; KCl, 0.3; $FeCl_2 \cdot nH_2O$, 0.01. A loopful of 5 days culture of the bacterial strains was inoculated to 500-m*l* flasks containing 50 m*l* of the basal salt solutions and 100 mg of the desulfurized fuel oil. The inoculated flasks were incubated at 25°C for 14 days on a reciprocal shaker. Since this medium contained no carbon sources other than the added fuel oil, growth and degradation of oil could easily be detected by visual inspection (*i.e.* turbidity and emulsification of oil). When a bacterial strain incapable of degrading oil was inoculated to this medium as a control, neither growth nor oil emulsificantion was observed. To recover the residual oil after bacterial degradation, cultures were extracted with *n*-hexane. An uninoculated control sample was extracted at the same time as the inoculated culture. The extract was dried over 10 g of Na_2SO_4 and concentrated by

rotary flash evaporator at 45°C to constant weight. Oil degrading activity of each strain was determined as % decrease of dry weight compared with the control sample.

Result

Table 1 presents the flora and average oil-degrading activity of oil-degrading bacteria isolated from various stations in oil-polluted Bisan Seto (sampling date: May, 1976). The levels of oil concentration, numbers of oil-degrading bacteria and total heterotrophs, which have been reported previously,[2,5] are also shown for reference. Neither numbers of oil-degrading bacteria nor the ratio of oil-degrading bacteria to total heterotrophic bacteria appeared to have a significant correlation with the concentration of oil. In this study, 90% of the oil-degrading bacterial strains isolated from various stations on this date was gram positive, catalase positive, nonmotile, nonsporeforming rods that have the characteristic of forming irregular-shaped cell arrangements. They were identified as coryneforms. Other isolates were also obtained from station 10 and station 12. These isolates were identified as *Moraxella*. They were incapable of making visible colonies on ZoBell 2216E agar medium. Experiments in ZoBell 2216E medium supplied with various additional carbon sources demonstrated that they were capable of making visible colonies on this medium with the addition of desulfurized fuel oil, *n*-hexadecane, or several intermediates in the pathway of *n*-hexadecane oxidation, *i.e.*, *n*-hexadecanol, palmitic acid, or acetic acid. They were found incapable of making visible colonies with the addition of glucose nor several organic acids (sodium citrate, sodium succinate, sodium malate). Oil-degrading activity of each strain isolated from various stations appears to be somewhat constant (about 20%) and there was no significant difference in the average of oil-degrading activity of the strains calcuclated on the basis of the stations where they were isolated.

Table 2 presents seasonal variation in the parameters described in Table 1 (sampling site: station 10). The concentration of oil in August and Nomvember were considerably lower than those in Febrary and May, suggesting self-purification process was promoted during this period. There was no significant difference in numbers of oil-degrading bacteria throughout the year. Dominant oil-degrading bacteria throughout the year was coryneforms. In May, *Moraxella* that were incapable of making visible colonies on ZoBell 2216E medium were also isolated. In August, 33% of the isolated strains was *Pseudomonas*. There were no significant difference in the average oil-degrading activity of the strains caluculated on the basis of the seasons when they were isolated.

Table 3 presents the overall comparison of the average values of the parameters between oil-polluted Bisan Seto and oil-unpolluted Hibiki

Table 1. Flora of oil-degrading bacteria, their average biodegradation of desulfurized fuel oil, and other parameters at various sampling stations in oil-polluted Bisan Seto (May 1976)

Parameter	Station No.								Total B
	1	2	4	6	7	9	10	12	
No. of isolates (% Total A)									
Coryneforms	6 (100)	10 (100)	5 (100)	7 (100)	1 (100)	2 (100)	8 ((73)	8 (80)	47 (90)
Moraxella spp.							3 (27)	2 (20)	5 (10)
Total A	6 (100)	10 (100)	5 (100)	7 (100)	1 (100)	2 (100)	11 (100)	10 (100)	52 (100)
Average oil degradation	20.1%	23.8%	20.7%	21.5%	29.7%	19.8%	26.8%	20.8%	22.3%
Oil-degrading bacteria (per m*l*)*	5.1×10^{0}	3.2×10^{0}	5.5×10^{1}	1.1×10^{0}	2.0×10^{1}	1.5×10^{0}	7.0×10^{1}	1.9×10^{1}	2.2×10^{1}
Heterotrophic bacteria (per m*l*)*	1.4×10^{4}	1.0×10^{4}	1.0×10^{3}	1.1×10^{3}	8.6×10^{3}	6.0×10^{2}	4.4×10^{4}	1.2×10^{4}	1.1×10^{4}
Oil content (ppm)*	0.48	0.09	1.0	0.08	0.08	0.13	0.48	0.24	0.32

* These prameters were previosly reported.[2,5]

Table 2. Seasonal variation in flora of oil-degrading bacteria, their average biodegradation of desulfulzed fuel oil, and other parameters in oil-polluted Bisan Seto sea waters (1976, st. 10)

Parameter	Sampling period				Total B
	February	May	August	November	
No. of isolates (% Total A)					
Coryneforms	7 (100)	8 (73)	10 (76)	7 (100)	32 (80)
Pseudomonas spp.			5 (33)		5 (13)
Moraxella spp.		3 (27)			3 (7)
Total A	7 (100)	11 (100)	15 (100)	7 (100)	40 (100)
Average oil degradation	22.8%	26.8%	16.8%	21.4%	21.2%
Oil-degrading bacteria (per ml)*	1.6×10^{1}	7.0×10^{1}	1.1×10^{2}	6.5×10^{1}	6.5×10^{1}
Heterotrophic bacteria (per ml)*	1.0×10^{4}	4.4×10^{4}	3.6×10^{5}	2.6×10^{3}	1.0×10^{5}
Oil content (ppm)*	0.40	0.48	0.30	0.15	0.28

* These parameters were previouly reported.[2,5]

Table 3. Comparison of flora of oil-degrading bacteria, their average biodegradation of desulfurized fuel oil, and other parameters in oil-polluted (Bisan Seto) and oil-unpolluted (Hibiki Nada) sea waters*1

Parameter	Unpolluted water (Hibiki Nada)	Polluted water (Bisan Seto)
No. of isolates (% Total A)		
Coryneforms	27 (54.0)	71 (87.6)
Pseudomonas spp.	23 (46.0)	5 (6.2)
Moraxella spp.	—	5 (6.2)
Total A	50 (100)	81 (100)
Average oil degradation		
Coryneforms	21.1%	22.1%
Pseudomonas spp.	5.9%	9.0%
Moraxella spp.	—	26.6%
Total average*2	14.1±8.8%	21.6±6.5%
Oil-degrading bacteria (per ml)*3	1.9×10^{1}	3.3×10^{1}
Heterotrophic bacteria (per ml)*3	3.4×10^{3}	4.2×10^{4}
Oil content (ppm)*3	0.11	0.31

*1 All figures were calculated from samples taken together (In Bisan Seto: May, 1976; st. 1, st. 2, st. 4, st. 6, st. 7, st. 10, st. 12: February, August, November, 1976; st. 10. In Hibiki Nada: March, June, October, 1976; st. B, st. C).

*2 Mean value±standard deviation.

*3 These parameters were previously reported.[2,5]

Nada. The average oil concentration in oil-oplluted Bisan Seto was 0.31 ppm and that of oil-unpolluted Hibiki Nada was 0.11 ppm. There was a slight difference in numbers of oil-degrading bacteria between two areas (3.3×10^{1} per ml in Bisan Seto, 1.9×10^{1} per ml in Hibiki Nada). A striking difference was found when the flora and oil-degrading potential of oil-degrading bacteria between two areas were compared. In Bisan Seto, coryneforms was an almost exclusively dominant (87.6%). In Hibiki Nada, coryneforms (54.0%) and *Pseudomonas* (46.0%) was isolated in the same ratio. The average oil-degrading activity of coryneforms isolated from Bisan Seto was 22.1% and that form Hibiki Nada was 21.1%. The average oil-degrading activity of *Pseudo-*

monas isolated from Bisan Seto was 9.0% and that from Hibiki Nada was 5.9%. The average oil-degrading activity of *Moraxella* isolated from Bisan Seto was 26.6%. When the average oil-degrading activity of the bacterial strains caluculated on the basis of areas were compared, that of Bisan Seto (21.6%) was significantly higher than that of Hibiki Nada (14.1%).

Discussion

In the last 20 years, there has appeared a large number of publications concerning the relationship of oil degrading bacteria and the concentration of oil in the environment. Although many reports[10,11] showed a direct correlation between the concentration of oil and the number of oil-degrading bacteria in water, other reports[12,13] showed that there was no such correlation. In our previous study,[5] it has been noted that there is no significant difference in numbers of oil-degrading bacteria between oil-polluted Bisan Seto and oil-unpolluted Hibiki Nada. In this study, it was noted that there was a significant difference between two areas, when the flora and the average oil-degrading activity of the isolated strains were compared. In oil-polluted Bisan Seto, coryneforms were almost exclusively dominant, whose oil-degrading activity was found to be much higher than that of *Pseudomonas*. These results suggested that coryneforms play an important role in oil-polluted Bisan Seto. It was also suggested that the self purification activity of oil-polluted Bisan Seto is significantly higher than that of Hibiki Nada.

In addition to the significant difference in the flora and the oil-degrading activity of oil-degrading bacteria, the other interesting microbiological characteristic was observed. In oil-polluted Bisan Seto, five strains of *Moraxella* were isolated, all of which were incapable of making visible colony on ZoBell 2216E agar medium unless oil, hydrocarbon, or their metabolic intermediates were supplied to this medium. These results indicate that their metabolism is highly adapted to hydrocarbon. At this time, a study on their nutritional physiology is not completed, but these results support the concept that a highly oil-polluted environment is composed of a distinctly unique microbial community structure.

The results obtained in this study suggest that the flora of oil-degrading bacteria and the oil-degrading activity of them are more sensitive indicators of oil pollution than other parameters previously reported (*i.e.* numbers of oil-degrading bacteria, the ratio of the degraders to the total heterotrophs).

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