Vertical Composition and Character Analysis of Saprophytic Bacteria Isolated from the Mudflat of Nakdong River Estuary

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洛東江 河口 干潟地에서 分離된 細菌의 層別 種組成 및 特性에 관하여

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Bacterial identification was performed with morphological, physiological and biochemical tests to the isolates from the mudflat of 30 cm depth sampled in Nakdong river estuary in March and June, 1985. *Flavobacterium* and *Enterobacteriaceae* were regarded as dominants. *Pseudomonas, Bacillus, Micrococcus, Vibrio, Aerococcus, Aeromonas, Acinetobacter* and *Staphylococcus* were founded in various depth. Vertical composition of bacterial genera in March was more diversiform than that of June. Character analysis was carried out with the calculation of similarity index (S). At a level of 85% similarity, the isolates were clustered into 5 groups and ungrouped 2 strains. Classifying groups of bacterial strains with determination schemes and groups from similarity index were in good agreement.

Bacterial role in natural ecosystem, especially intertidal sediment is regarded as nutrient regeneration, energy transfer and introduction of organic matter to higher trophic levels. But these roles of bacteria were not understood precisely well. Studies have approached to comprehend remineralization cycle in anoxic state of sediment and population dynamics in surface layer. Whereas the accumulation of organic matarials in sediment from input of organic substance endow the role of heterotrophic bacteria with significant meaning. From this viewpoint researches on composition of heterotrophic bacteria are needed to understand the function of aerobic heterotrophs in estuarine sediments.

Research area was located in the tidal mudflat near sand bar, Okryudeung, one of the well-developed delta and sand bars in Nakdong river. The tidal flat have been influenced with Nakdong river and South Sea and many topographical changes have been occurred. Okryudeung as sand bar has been formed through past *ca.* 65 years. In 1982, the area of Okryudeung was measured 615,000 m^2. The active formation of many sand bars and deltas in Nakdong river estuary had began after the construction of watergate at Jukrim stream in 1934. Also the destruction of forest at upper area in Nakdong river had influence on the formation of those. And the research area maintained the eutrophicated state with input of a large quantity of various nutrients from Pusan city and high primary productivities were measured (Hong, 1984). These conditions of research area gathered passage birds near deltas and many sand bars in the winter seasons. A few biological reports of the research area were published; reports on the observation of 136 spp. of bird (Won, 1974), on the development of plant

community in sand bar (Moon, 1984), on fish fauna of Nakdong river (Kim and Hong, 1980), on the structure and function of estuarine ecosystem (Kim *et al.*, 1982) and on the presence and ecology of invertebrate community (Kim *et al.*, 1979).

In Nakdong river the watergate for the elimination of seawater effects has been in course of construction. After the construction of dam the whole ecosystem of estuary may be affected in native structure and function. To understand the effects of dam construction to native ecosystem, more efforts to obtain the basic informations are required.

Microbiological studies in mudflat were carried out mainly with the population dynamics and remineralization processes in surface layer. But studies of bacterial taxonomy and vertical compositions of bacteral genera for comprehension of structure and function of ecosystem were scarcely reported. Especially character analysis from physiological and biochemical specificities of bacterial isolates provide the important informations for understanding of function of bacteria as microconsumer in natural ecosystem.

MATERIALS AND METHODS

Sampling site

The pH of sampling sites was in the range of 6.0-7.0 and sand, clay and silt consisted Okryudeung. Among these components, sand was a major component of Okryudeung. The total number of plant species present in Okryudeung was 88, vegetation types of sampling area were divided into two groups: mixed plant community of sand dune vegetation dominated by *Cynodon dactylon, Carex pumila, Calystegia soldanella, Lathyrus japonica* and *Oenothera odorata* and salt marsh vegetation dominated by *Carex scabrifolia, Phragmites communis,* and *Zoysia sinica* (Moon, 1984). The regions around sand bars were used for nurseries of *Corbicula japonica* Prime, many kinds of passage birds have immigrated to sampling area in winter seasons. The site M was located near the community of *Phragmites communis,* relatively muddy, wet. The site S was located in sand beach. When there is on the ebb tide, sampling sites appear to the atmosphere. The site S appeared first, the site M late. Therefore, the submergence time of the site M was longer than that of the site S. Mudflat samples were obtained after ebb (Figure 1).

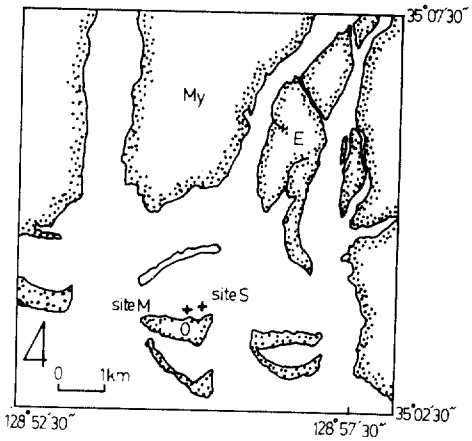

Fig. 1. *Map of the research area and sampling sites (S, M) in mudflat near the sand bar of Okryudeung in Nakdong river estuary (E; Eulsukdo, My; Myeonghodo, O; Okryudeung).*

Table 1. *Occurrence of saprophytic bacteria isolated from mud flat.*

Identified group	30 March, 1985		1 June, 1985	
	Site M	Site S	Site M	Site S
Enterobacteriaceae	+	+	+	+
Flavobacterium	+	+	+	+
Pseudomonas	+	+	+	+
Bacillus	+	+	+	+
Micrococcus	+		+	+
Vibrio	+	+	+	
Aerococcus			+	+
Aeromonas		+	+	
Acinetobacter	+	+		
Staphylococcus	+			

Sampling procedures

Mudflat samples were collected with PVC sediment corer (10cm diameter, 50cm length) in the seasons listed in Table 1. Thirty centimeter-long core samples were sliced up for 1cm to 10cm in depth, 2cm to the 20cm. Sliced samples were collected in sterilized specimen cup (Green Cross Medical Equipment Co. Seoul). For bacteriologi-

cal analysis, 2ml of samples was obtained from the original mass of samples with tip-cut syringe aseptically to 198ml of sterile isotonic water as dilution water. Samples were homogenized with the aid of a Virtis 45 homogenizer (The Virtis Co. N.Y., USA) for 5 min.

Isolation

ZoBell's 2216e agar medium (Oppenheimer and ZoBell, 1952) with the distilled water was used for development of heterotrophic bacteria. Serial dilutions of the homogenized mudflat samples were poured into the plate with agar medium. The inoculated petri dishes were incubated in the dark for 7 days at 25°C. Developed colonies were enumerated with the aid of a Harris counter CC 30 (Philip Harris Ltd. Shenstone, England). For the pure isolation, colonies were inoculated to fresh medium and single colony was obtained after 2 days incubation at 25°C. This procedure was carried out two times. The pure isolates were conserved in stab culture with ZoBell's 2216e medium slant.

Taxonomy

Identification of pure isolates was performed by the methods described from Shewan *et al.*, (1960). The physiological tests were accepted with reference to Bergey's manual of systematic bacteriology (Krieg and Holt, 1984), Manual for the identification of medical bacteria (Cowan, 1974) and Biochemical tests for identification of medical bacteria (MacFadin, 1980).

Character analysis

Percentage of similarity was obtained from the results of morphological, physiological and biochemical tests. The calculation of the similarity index was carried out according to the eqution;

$$100 \times \frac{N_s}{N_s + N_d} = S\ (\%)$$

where N_s respresents the number of characters shared by two strains and N_d the number of characters differing between two strains (Sneath, 1957).

The clustering methods follow the single linkage clustering: two strains or two groups are linked together with the highest S values which is shared by two members of these groups (Sneath and Sokal, 1973).

RESULTS

Temperatures of 2cm depth from surface were measured 6°C in S site and 8°C in the site M, air temperatures measured at the same time were 5°C and 7.2°C respectively in March. In sampling of June, temperature of both sites was 21°C whereas air temperature was the range of 17°C to 22°C. Thirty centimeters of core sample of the site S was composed of: 0-5cm brown region; 5-13cm grey region; 13-18cm black; 18-20cm grey; 20-30cm black in March and composed of: 0-9cm brown; 9-18cm black; 18-30cm brown in June. Profile of M site sample in March was composed of: 0-4cm brown; 4-16cm brownish black; 16-30cm black and in June: 0-4cm brown; 4-9cm grey; 9-30cm black (Fig. 2).

About 2cm of surface in the sampling sites was eroded away by the tidal effect during 9 months after Sep. 1984. Identified bacterial genera present in research area were listed in Table 1. Ten genera were identified in research area; *Enterobacteriaceae, Flavobacterium, Pseudomonas, Vibrio, Bacillus, Staphylococcus, Acinetobacter, Micrococcus, Aerococcus* and *Aeromonas*. Dominant genera were *Flavobacterium, Bacillus, Pseudomonas* and *Enterobacteriaceae*.

In the site M, all of ten genera were found; *Flavobacterium, Enterobacteriaceae, Bacillus, Pseudomonas, Micrococcus* and *Vibrio* were isolated in March and June. *Staphylococcus* and *Acinetobacter* were identified only in March, *Aerococcus* and *Aeromonas* in June. *Flavobacterium, Bacillus,*

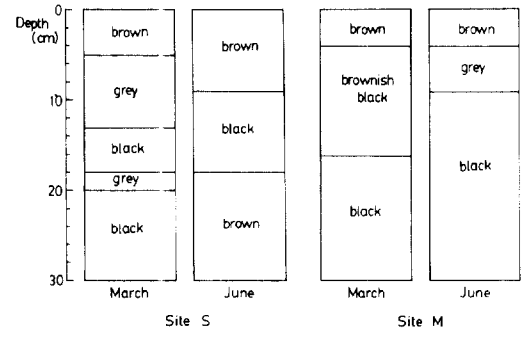

Fig. 2. *Diagrams showing the characteristics of colours in the sampled cores.*

Table 2. *Vertical composition of bacterial flora identified (% of populations in each depth).*

March

	depth (cm)	Flavo.	Bacil.	Entero.	Pseudo.	Micro.	Staph.	Acineto.	Vibrio
M	1	27.0	–	27.0	45.9	–	–	–	–
	2	8.3	–	–	50.0	–	41.7	–	–
	3	–	–	93.3	–	–	6.7	–	–
	4	–	–	–	–	–	–	–	–
	5	–	1.8	–	98.2	–	–	–	–
	6	–	23.1	–	76.9	–	–	–	–
	7	–	41.7	41.7	16.7	–	–	–	–
	8	18.2	18.2	–	54.5	–	–	9.1	32.8
	9	16.4	–	–	34.4	16.4	–	67.6	–
	10	28.4	–	4.1	–	–	–	67.6	–
	11	17.4	39.1	–	–	43.4	–	–	–
	13	40.0	20.0	40.0	–	–	–	–	–
	15	100.0	–	–	–	–	–	–	–
	17	35.7	64.3	–	–	–	–	–	–
	19	12.5	–	–	25.0	–	12.5	12.5	37.5
	21	–	6.8	45.5	2.3	22.7	22.7	–	–
	23	35.7	14.3	3.6	–	35.7	–	–	10.7
	25	5.8	13.3	–	23.1	57.8	–	–	–
	27	100.0	–	–	–	–	–	–	–
	29	88.3	–	–	6.0	–	–	–	–
Total		29.3	22.2	17.2	13.9	10.2	2.9	2.4	1.8

	depth (cm)	Flavo.	Pseudo.	Entero.	Bacil.	Vibrio	Acineto.	Aeromo.
S	1	19.0	–	71.2	9.8	–	–	–
	2	47.6	–	40.2	12.2	–	–	–
	3	41.7	34.4	24.0	–	–	–	–
	4	75.0	7.1	17.9	–	–	–	–
	5	89.6	–	10.4	–	–	–	–
	6	2.9	90.6	6.5	–	–	–	–
	7	89.8	–	3.3	6.9	–	–	–
	8	5.2	90.4	–	4.3	–	–	–
	9	5.7	34.3	31.4	–	28.6	–	–
	10	–	87.3	12.7	–	–	–	–
	11	21.2	29.5	34.2	14.1	–	–	–
	13	51.0	16.8	2.0	30.2	–	–	–
	15	–	37.0	28.3	–	–	34.8	–
	17	78.2	3.6	18.2	–	–	–	–
	19	80.4	6.0	–	13.7	–	–	–
	21	40.5	27.0	–	32.4	–	–	–
	23	2.1	77.1	–	20.8	–	–	–
	25	–	38.3	–	58.3	–	3.3	–
	27	9.4	47.2	20.8	–	18.9	–	3.8
	29	8.7	91.3	–	–	–	–	–
Total		44.4	25.4	15.9	12.6	0.9	0.8	0.2

June

	depth (cm)	Entero.	Pseudo.	Flavo.	Bacil.	Aeroco.	Micro.	Aeromo.	Vibrio
M	1	31.8	22.7	–	–	–	–	22.7	22.7
	2	17.9	–	–	35.7	–	39.3	7.1	–
	3	90.9	4.5	4.5	–	–	–	–	–
	4	50.0	32.3	17.7	–	–	–	–	–
	5	–	71.4	28.6	–	–	–	–	–
	6	45.7	47.8	2.2	2.2	2.2	–	–	–
	7	52.4	–	–	47.6	–	–	–	–
	8	100.0	–	–	–	–	–	–	–
	9	86.1	8.3	–	2.8	–	2.8	–	–
	10	91.3	–	–	4.3	–	4.3	–	–
	11	–	52.0	–	48.0	–	–	–	–
	13	94.1	–	–	5.9	–	–	–	–
	15	96.0	1.3	0.7	0.7	1.3	–	–	–
	17	10.7	50.0	35.7	–	–	3.6	–	–
	19	93.8	3.1	–	3.1	–	–	–	–
	21	47.6	4.8	–	–	47.6	–	–	–
	23	13.0	43.5	43.5	–	–	–	–	–
	25	46.5	27.9	23.3	–	2.3	–	–	–
	27	67.6	2.9	–	–	29.4	–	–	–
	29	25.8	9.7	–	–	64.5	–	–	–
Total		53.1	19.1	7.8	7.5	7.4	2.5	1.5	1.1

	depth (cm)	Entero.	Pseudo.	Flavo.	Bacil.	Aeroco.	Micro.
S	1	25.6	46.5	23.3	–	2.3	2.3
	2	98.5	1.5	–	–	–	–
	3	40.0	60.0	–	–	–	–
	4	–	90.9	9.1	–	–	–
	5	35.5	–	64.5	–	–	–
	6	–	39.3	–	–	60.7	–
	7	81.5	18.5	–	–	–	–
	8	40.0	–	10.0	10.0	40.0	–
	9	–	7.7	–	76.9	15.4	–
	10	50.0	–	–	50.0	–	–
	11	25.5	5.1	2.1	66.0	–	2.1
	13	–	70.0	30.0	–	–	–
	15	77.9	–	22.1	–	–	–
	17	94.6	0.7	4.7	–	–	–
	19	33.3	50.0	16.7	–	–	–
	21	45.5	–	18.2	–	27.3	9.1
	23	75.0	25.0	–	–	–	–
	25	47.6	47.6	4.8	–	–	–
	27	100.0	–	–	–	–	–
	29	75.0	–	25.0	–	–	–
Total		47.3	23.1	11.5	10.1	7.3	0.7

M; Site M
S; Site S

Acineto.; *Acinetobacter*
Aeroco.; *Aerococcus*
Aeromo.; *Aeromonas*
Bacil.; *Bacillus*
Entero.; *Enterobacteriaceae*
Flavo.; *Flavobacterium*
Micro.; *Micrococcus*
Pseudo.; *Pseudomonas*
Staph.; *Staphylococcus*

Enterobacteriaceae and *Pseudomonas* were dominant genera, whereas *Micrococcus, Staphylococcus, Acinetobacter* and *Vibrio* were found relatively few in March; in June *Enterobacteriaceae* and *Pseudomonas* were identified as dominants and *Flavobacterium, Bacillus, Aerococcus, Micrococcus, Aeromonas* and *Vibrio* were regarded as relative minor.

In the site S, nine genera similar to the site M were identified. *Flavobacterium, Pseudomonas, Enterobacteriaceae* and *Bacillus* were dominant bacterial groups respectively in March and *Enterobacteriaceae, Pseudomonas* and *Flavobacterium* found as dominant genera in June. Especially, *Flavobacterium* of the site M in March and *Enterobacteriaceae* of both sites in June had high population sizes to about one half of bacterial flora.

The influence of depth on the vertical distribution of bacterial flora was shown in Table 2. In the site S of March, the percentage of *Enterobacteriaceae* decreased 71.2% of surface layer to 3.3% of 7cm depth and random occurrence with the range of 34.2% to 2.0% was observed in the other depth. *Flavobacterium* was found in the whole depths, increase of 19.0% to 89.8% was shown in surface layer to 7cm depth (except 6cm layer) and another peak was 80.4% in 19cm layer. *Pseudomonas* was found generally also. The regions of 6cm to 15cm and of 21cm to 29cm layer had the population sizes above 16.8%. Six centimeter, 8cm, 10cm and 23cm layer had large quantities above 77.0%.

Whereas in the site M of March, *Enterobacteriaceae* was found in various depths in the range of 3.6% to 93.3%. From surface layer to 9cm depth, *Pseudomonas* was identified for the quantities of 16.7% to 98.2% range. Vertical distribution of *Flavobacterium* was in the range of 5.8% to 100%, surface to 2cm depth had 8.3% to 27.0% and in layers below 8cm were shown the occurrences of *Flavobacterium* in the range of 5.8% to 100%. In two centimeter, 3cm, 19cm and 21cm layers, *Staphylococcus* was found in 6.7% to 41.7% quantities.

Vertical distribution of bacterial flora in June was simpler than in March. Indentified genera were 6 in the site S, 8 in the site M. In the site M, almost all of depths (except of 5cm and 11cm layers) had *Enterobacteriaceae* as dominant in the range of 10.7% to 100% at the average of 53.1%. *Pseudomonas* had relatively a large portion of bacterial flora in 1.3% to 71.4%. In the site S, similar distribution to the site M was observed. Percentage of *Enterobacteriaceae* was 25.5% to 100% from the depths. In the layers below 15cm depth, relatively high population sizes were obtained above 33.3%. *Bacillus* was observed in 8cm to 11cm layer only with 10.0% to 76.9%.

Results from the physiological, morphological and biochemical tests for bacterial identification can be used not only for taxonomic analysis, but

Table 3. *Characteristics of bacterial flora isolated in March and June (% of populations).*

	March	June
MORPHOLOGICAL:		
rod shaped	93.4	93.5
coccus shaped	6.6	6.5
gram negative	76.0	82.3
gram positive	24.0	17.7
motile	34.8	41.2
nonmotile	65.2	58.8
pigmented	56.5	70.6
spore forming	15.2	11.8
PHYSIOLOGICAL:		
inositol	–	5.9
arginine	–	41.2
starch	8.7	29.4
MR	–	11.8
0/129 sensitive	–	88.2
gas production from glucose	0.0	0.0
fermentative	76.1	52.9
ENZYMATIC:		
catalase	100.0	100.0
oxidase	43.5	41.2
amylase	2.9	24.6
lipase	4.4	7.6
protease	3.7	43.5

also ecologically to describe microbial communities (Kaneko *et al.*, 1978). Characteristics of microbial flora isolated in both sites were listed in Table 3. Gram negative bacteria were found 76% in March, 82.3% in June. Rod shaped bacteria were shared in the portion of 93.4% and 93.5%. Physiological groups of bacteria with amylase, lipase and protease were increased from 2.9% to 24.6%, 4.4% to 7.6% and 3.7% to 43.5% respectively in March to June. No bacteria were gas productive from glucose.

At a level of the similarity index (S) above 85%, the isolates were divided into 5 groups and 2 ungrouped strains (Fig. 3). Similarity index means the relationships of morphological, physiological and biochemical characters among the each strains (Colwell and Liston, 1961; Bölter, 1977). Therefore, groups from similarity index may correspond to classifying phena of taxonomic analysis. Divided groups were compared to identified genera. In group 1, *Aeromonas, Vibrio, Pseudomonas, Enterobacteriaceae* and *Acinetobacter* were clustered together. *Flavobacterium* spp. were clustered into two groups; group 2 and group 3. *Bacillus* spp. were linked together as group 4. Group 5 consisted of *Staphylococcus, Aerococcus* and *Micrococcus* and ungrouped 2 strains were identified to *Aerococcus.*

At a level of S above 75%, isolates were divided into larger 3 groups. First group was identical with group 1 described above, second group was consisted of group 2 and group 3. Final group was clustered with group 4, group 5 and ungrouped strains. First and second groups were clusters of gram negative bacteria, strains of final group were linked together with the aspect of gram positive bacteria.

DISCUSSIONS

The bacterial distribution in sediment of freshwater and marine was not well understood. Number of saprophytes was measured in various sedimental ecosystem and in the surface of sediment, also physiologically specific bacterial populations (e.g., sulfur-reducing bacteria) were determined for mineralization rates, *etc.* (Exerzew, 1948; Kusnezow, 1959; Suzuki, 1961; Dale, 1974; Boeyé *et al.*, 1975; Litchfield *et al.*, 1975; Karl, 1978; Rieper, 1979; Aller and Yingst, 1980; Jones, 1982). Taxonomic analysis of bacteria in sediment habitats were very few, especially vertical distribution of bacteria was scarcely reported (Kaneta and Colwell, 1974· Boeyé *et al.*, 1975; Kwon, 1983).

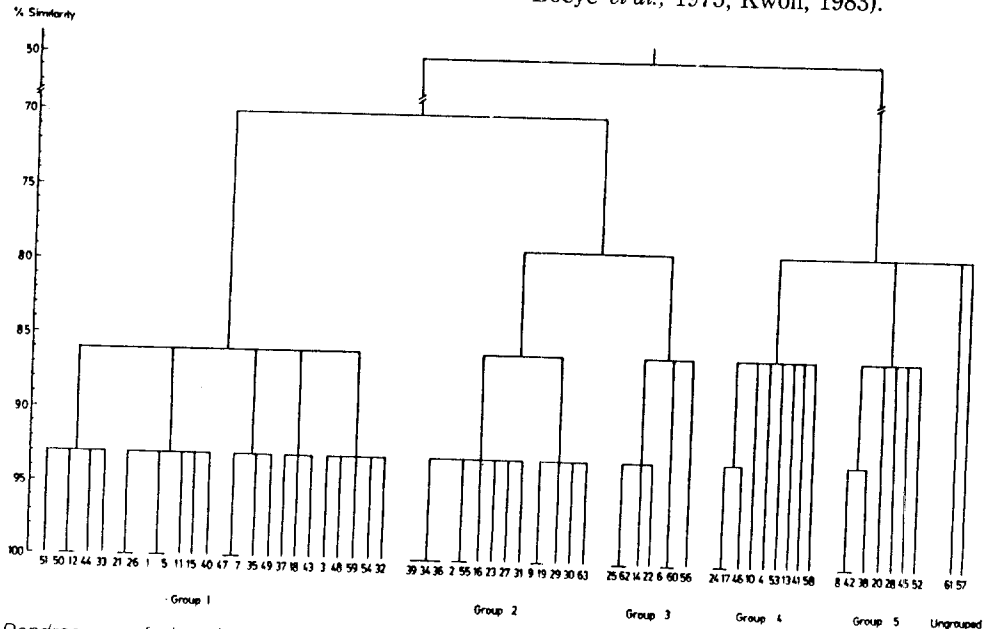

Fig. 3. *Dendrogram of the characters in the isolated bacteria from mud flat.*

Enterobacteriaceae in research area was regarded as a major dominant group, especially, in June (47.3%-53.1%). This might be resulted from the location of sampling sites and the increase of temperature. Sampling sites located in a downstream of wastewater treatment facilities of domestic sewage from Pusan city. Enteric bacteria could be introduced to the research area in a large quantity from the facilities' output. Enteric bacteria survive only for a short time in marine aquatic environment. The rapid decrease of enteric bacteria in marine water column is caused by various factors: biological, physical and chemical factors (Rittenberg et al., 1958; Carlucci and Pramer, 1960 a,b; Sieburth and Pratt, 1962; Jones, 1964; Gameson and Saxon, 1967; Enzinger and Cooper, 1976). Compared to rapid decline of enteric bacteria in water, the longer survival time of enteric bacteria was observed in sediment or soil environments (Gerba and McLeod, 1976; Papavassiliou and Leonardopoulos, 1978; Bauerfeind *et al.*, 1981). One of the major factors of that might be thought to be the high content of available organic substances. These might explain that the existence of *Enterobacteriaceae* as enteric bacteria was observed in dominant group through the whole depths.

Gram negative bacteria measured in North Sea sediment was 40.1% (Boeyé *et al.*, 1975) and in Jinhae Bay sediment 56.3% (Kwon, 1983). In our research area, gram negative bacteria were in the range of 76.0%-82.3%. And rod shaped bacteria were found a larger portion than those in North Sea and Jinhae Bay. But all of the bacteria isolated from sediment of subarctic marine were identified to rod shaped, gram negative bacteria (Kaneko *et al.*, 1978).

Some specific enzymatic activities were compared to results of Kaneko *et al.*, (1978), bacteria with lipase in subarctic environment were *ca.* 15 times as those of this study. Amylase producing bacteria in March of research area were 2.9% and 24.6% in June. Amylolytic and proteolytic bacteria were smaller than those of arctic and subarctic sediments.

Lipolytic, amylolytic and proteolytic bacteria in June were increased to 1.7, 8.5 and 11.8 times as those in March. These might be influenced with temperature, which was 6°C-8°C in March and 21°C in June. Similar effects were observed in other measured bacteriological parameters. Bacterial heterotrophic potential measured with uptake rate of ^{14}C-glucose were increased considerably and also number of saprophytes was increased for 1-2 orders of magnitude in the same period (unpublished data).

Vertical composition of bacterial genera in March was more diversiform than that of June whereas quantitative increase of saprophytes was observed March to June. Hauxhurst *et al.*, (1981) hypothesized that there would be an inverse relationship between population size and taxonomic diversity; high population sizes should reflect competitive success of a limited number of population.

Character analysis was performed for clustering of physiologically related bacterial types. Classifying groups of bacterial strains with determination schemes and groups from similarity index were in good agreement at a level of 85% similarity. The fluctuation of the physiologically specific bacteria is more important than that of taxonomic groups. The physiologically specific bacteria may indicate the heterotrophic activity. From character analysis, it becomes possible to describe the characteristics of bacterial population in sediment.

Understanding bacterial composition in the profile of mudflat considerably contributes to the comprehension of remineralization process as a major role of microorganisms and the structure of ecosystem.

摘 要

1985年 3月과 6月에 洛東江 河口의 干潟地에서 30cm 깊이로 採集한 堆積土壤標本에서 細菌을 分離하였다. 分離된 細菌으로 形態学的, 生理学的, 生化学的 実験을 통해 系統分類하였다. *Flavobacterium*과 *Enterobacteriaceae* 가 주된 優点種으로 나타났으며, *Pseudomonas*, *Bacillus*, *Micrococcus*, *Vibrio*, *Aerococcus*, *Aeromonas*,

Acinetobacter, *Staphylococcus*가 각 깊이별로 多樣한 組成을 보였다. 계절에 의한 뚜렷한 種組成의 変化를 觀察하였다.

分離된 細菌의 여러 特性을 similarity index를 利用하여 菌株別 連関性을 分析하였다. 分離된 細菌은 다섯개의 集団과 그에 속하지 않는 두 종의 菌株로 나뉘었다. 統計学的 集団과 系統分類된 集団사이에는 높은 類似性이 있음을 보았다.

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