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Effects of Acidification on the Changes of Microbial Diversity in Aquatic Microcosms

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In an artificial pH-gradient batch culture system, the effects of acidification on the species composition of a heterotrophic bacterial community were analyzed. As a result of this study, it was found that total bacteria numbers were not affected by acidification and that the population of heterotrophic bacteria decreased as pH became lower. The heterotrophic bacteria isolated from the entire pH gradient were 12 genera and 22 species. Among them, 64% were gram negative and 36% were gram positive bacteria. As pH decreased, the distribution rate of gram negative bacteria increased while that of gram positive bacteria decreased. The diversity of genera decreased from 13 to 5 as pH decreased from 7 to 3. The G+C content of all of the 202 isolated strains varied from 22.8 to 77.0%, and increased in interspecies of same genus as pH decreased. As a result of clustering analysis, the diversity index of species ranged from 1.13 to 2.37, and it had lower indices as pH decreased. In order to evaluate the diversity of numbers of sample of different size, a rarefaction method was used to analyze the expected number of species appearance according to pH. The statistical significance of species diversity was verified by the fact that the number decreased at lower pH.

According to An International Collaborative Project on Acid Rain in Asia (Carmichael et al., 1994), it is expected that the amount of sulfur dioxide emitted from Asia will exceed that of North America and Europe after the year 2010. Air pollution in Northeast Asia including Korea has reached a level similar to West Europe and North America, and it is certain that this area will be the largest air pollutant-emitting zone in the near future. China is producing more than 78% of the sulfur dioxide emitted in this area (Wang, 1993). The effluent sources of pollutants are concentrated in the Yellow Sea shores of mainland China and those pollutants are carried by the prevailing westerlies. Korea and Japan, which are located in the direction of this wind, are expected to be greatly affected (Kang et al., 1993; Hadakeyama, 1995).

Substantial research is being done in Korea concerning the movement of pollutants from neighboring countries including China as well as from domestic sources (Kim et al., 1989; Kim et al., 1990; Lee et al., 1995). Studies on the influence of acid rain are also in process (Chang et al., 1990; Lee, 1996). However, compared to studies on territorial ecosystems such as forestry and agriculture, studies of aquatic ecosystems have been rather unsatisfactory (Ahn et al., 1995). This is due to the fact that the pH of most Korean lakes, which are undergoing or have already undergone eutrophication, is easily changed from neutral to alkaline by the influence of seasonal algal blooms (Kim et al., 1996). No visible damage by aquatic acidification has been reported in Korea until now. However, as serious damage by aquatic acidification does not appear quickly, but slowly by the effects of accumulated acidic precipitation (Howells, 1995), studies forecasting the effects of acidification on aquatic ecosystems are a very important subject in Korea.

Since 1970, typical studies concerning the influences of aquatic acidification on microbial ecosystems have been done in three ways. First, a study comparing acidified areas and non-acidified areas (Osgood, 1987; Stokes et al., 1989; Rattray and Logan, 1993). Second, a study monitoring changes in the ecosystem of specific areas in the process of acidification (Harvey, 1989; Schindler, 1994). Lastly, studies on the degrading ability of organic compounds with various pH values by using microcosm (McKinley and Vestal, 1982; Matschullat and Wyrobek, 1993). In Korea, owing to the regional characteristics of freshwater, acidifiable lakes have not been reported except in local areas directly affected by acid mine drainage.

Since March 1994, we have studied the relationships

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between physicochemical parameters such as pH and microbial ecosystems at reservoirs in industrial areas near big cities (Ahn et al., 1995). As Wangsong Reservoir is located not only in the middle of an industrial park, but in the western region of Korea, it can be considered representative of those areas affected by acid precipitation carried by prevailing westerlies from China. For the past 3 years (1994-1996), the pH of this reservoir has varied seasonally from 6.02 to 10.24, and no acidification tendency was found. Multiple regression analysis shows that changes in variable pH correlated with heterotrophic and total bacteria populations implying that the pH greatly affected species composition and microbial functions in this area (Ahn et al., 1995).

In this study, the influence of acidification on the species composition and diversity index of heterotrophic bacteria with an artificial pH gradient batch culture system was analyzed. Regarding the strains isolated from each pH section, we did cluster analysis based on 43 morphological and biochemical characteristics and analyzed the G+C content of the isolates according to pH.

Materials and methods

Sampling and batch culture system

In June 1996, water samples were collected from the Wangsong Reservoir (lat. 36° 18′ N., long. 126° 55′ E.) in Kyongido, a region near Seoul. To make a batch culture system, each 1.5-liter water sample in sterilized glass jars with covers were adjusted to pH 3.0, 4.0, 5.0, 6.0, and 7.0 with 2N H_2SO_4. The samples from each jars were analyzed every 48 h over a 10 d incubation at 25℃.

Measurement of the physical and chemical factors

Environmental factors of samples from each batch were analyzed according to its pH, dissolved oxygen, temperature, conductivity, and turbidity which were measured by a water quality analyser (Horiba U-10, Horiba, Japan).

Water samples were passed through membrane filters (Gelman, 47 mm, pore size 0.45 μm), then amounts of ammonia, nitrite, and phosphate were measured according to the method of the American Public Health Association (1992). Soluble sugar was measured by the Anthrone method (Herbert et al., 1971) and then its amount was converted into glucose as a standard value. Total organic matter was measured by the Walkley method (Herbert et al., 1971).

Microbiological analysis

Water samples were shaken vigorously to disperse clumps and fixed with buffered formalin (final concentration 4%). Acridine orange direct counts (AODC) were made by the method of Hobbie et al. (1977). Nuclepore polycarbonate filters (Gelman, 25 mm, pore size 0.2 μm) were prestained with Sudan Black B. The fixed water sample (1 ml) was mixed with 1.5 ml of acridine orange solution (1:10,000 in 6.6 mM phosphate buffer, pH 6.7). After 5 min, the sample was filtered through the prestained filter. The mounted filter was examined and counted with an Epifluorescence microscope (Axioplane, Ziess, Germany). At least 20 fields were examined per filter.

Numbers of viable heterotrophic bacteria were estimated by spreading 0.1 ml amounts of samples diluted 10^{-2} in distilled water on 10% nutrient agar in triplicate. After incubation at 25℃ for 7 d, the colonies were counted. 10% nutrient agar consistently gave higher counts and more diverse populations than nutrient agar (Difco), trypticase soy agar (Difco), 5% trypticase soy agar, R2A agar (Difco) and casein peptone starch agar.

Isolation and identification of heterotrophic bacteria

For isolation of heterotrophic bacteria from each batch with pH 3, 4, 5, 6, and 7, the samples were diluted serially and inoculated on 10% nutrient agar plates adjusted to pH 3, 4, 5, 6, and 7 with 1N H_2SO_4. After incubation at 25℃ for 7 d, all colonies were collected from the 40-50 colony-forming agar plate. The isolates were tested by gram staining and their biochemical characteristics were identified with a gram negative identification (GNI) card and gram positive identification (GPI) card. The isolates were identified by Vitek^R system (BioMerieux, France) data base.

Measurement of G+C content

DNA was prepared and purified from a single colony by the NaOH method (Mesbah et al. 1989). To obtain the G+C content, DNA was degraded with P1 nuclease (1 mg · ml^{-1} in sodium acetate buffer; 340 U · ml^{-1}) and bovine intestinal mucosa alkaline phosphatase (200 U · ml^{-1} in glysine buffer) to nucleosides as described by Mesbah et al. (1989). Then a sample containing between 0.5 and 1.5 μm of nucleosides was injected into a high performance liquid chromatograph (Waters 510, USA) with an Econosphere C18 reversed-phase column (400 × 4.6 mm). The solvent contained 12% methanol and 20 mM triethylamine phosphate (pH 5.1). The mole percent of G+C of each bacterial isolate was calculated from the nucleoside ratio as described by Mesbah et al. (1989). *Micrococcus luteus, Escherichia coli, Clostridium perfringens,* and calf thymus (Sigma) were used as standard strains. The G+C contents of these standard strains were 72 %, 50 %, 26.5 %, and 43 %, respectively.

Cluster analysis

Physiological profiles of each isolate for the variables of cluster analysis were obtained from the Vitek^R system

Table 1. Test parameters for character analysis of isolated bacteria

Morphology cell shape, Gram stain, colony morphology, motility
Physiological characteristics growth at 4, 37, 42℃
Biochemical characteristics catalase production, bile-aesculine hydrolysis, indole formation, MR test, VP test, nitrate reduction, OF test, oxidase production, urease production, H2S production, β-galactosidase production, polymyxin B sensitivity
Substrate utilization arginine hydrolysis, gelatin liquefaction, malonate utilization, starch hydrolysis, lysine hydrolysis, citrate utilization, ornithine hydrolysis, p-coumaric acid utilization, tryptophan utilization, acetamide utilization, plant indican utilization, DP-300 utilization
Carbohydrate fermentation adonitol, arabinose, glucose, inositol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, sorbitol, sucrose, xylose

(BioMerieux, France) card, the identification scheme of Biochemical tests for identification of medical bacteria (Macfaddin, 1980) and from Bergey's manual of determinative bacteriology (John et al., 1994) (Table 1). The physiological, morphological, and biochemical characters of 43 different profiles were coded in binary form (positive results and growth, 1; negative results and nongrowth, 2).

Similarity coefficients for each isolate comparing all other isolates were calculated by using the simple matching coefficient (S_{SM}) which resulted in a similarity index matrix. A dendrogram was made with the result of cluster analysis by using the unweighted average linkage method [UPGMA] (Rohlf 1993).

Diversity index

In order to compare the change of species diversity in a microbial community according to pH, the diversity index (H′) of each batch was calculated. The richness index, diversity index, and evenness index were calculated by the Menhinick index (R2), Shannon index (H′), and Pielou index (E1), respectively (Ludwig and Reynolds, 1988). The expected number (E(Sn)) of any species or group was calculated by applying the rerefaction method to standardize the number of samples with different size. Statistical evaluation of the rarefaction curves was made by inspection of the 95% confidence bands around the curves (Wassel and Mills, 1983).

Results

Physico-chemical environmental parameter and bacterial population

The variations in the environmental parameters and numbers of bacterial populations from batch culture systems with a pH gradient are summarized in Table 2. The concentration of total organic matter, soluble sugar, and turbidity decreased and ammonia and conductivity increased as pH became lower. Numbers of total bacteria and heterotrophic bacteria ranged

Table 2. Physico-chemical factors and bacterial population from each batch

Factor	Adjusted pH at				
	7	6	5	4	3
pH	7.49 ±0.51[a]	5.88 ±0.64	4.88 ±0.20	4.19 ±0.19	3.09 ±0.16
Conductivity (mS · Cm^{-1})	0.48 ±0.21	0.52 ±0.17	0.57 ±0.09	0.57 ±0.07	1.02 ±0.22
Turbidity (NTU)	124 ±13.2	130 ±23.1	73 ±9.12	55 ±5.01	34 ±5.11
TOM (mg · ℓ^{-1})[b]	517.0 ±5.83	612.2 ±6.32	557.8 ±5.93	467.2 ±5.17	476.2 ±4.52
SS (mg · ℓ^{-1})[c]	0.74 ±0.08	0.79 ±0.06	0.91 ±0.03	0.87 ±0.02	0.77 ±0.01
PO_4 (μg · ℓ^{-1})	0.42 ±0.13	0.52 ±0.14	0.37 ±0.07	0.51 ±0.02	0.30 ±0.05
NO_2 (μg · ℓ^{-1})	0.037 ±0.005	0.031 ±0.007	0.019 ±0.006	0.021 ±0.005	0.015 ±0.007
NO_3 (μg · ℓ^{-1})	318.2 ±59.3	257.9 ±65.5	134.8 ±42.2	126.1 ±33.4	108.1 ±25.5
NH_4 (μg · ℓ^{-1})	0.01 ±0.001	0.13 ±0.002	2.03 ±0.09	2.32 ±0.13	2.27 ±0.17
Total bacteria (10^6 cells · ml^{-1})[d]	2.42 ±0.75	1.24 ±0.89	1.05 ±0.78	0.77 ±0.35	0.71 ±0.24
Heterotrophs (10^5 cfu · ml^{-1})[e]	6.27 ±1.32	2.23 ±1.15	0.89 ±0.77	0.71 ±0.51	0.13 ±0.09

[a] $\bar{\chi}$ ±SD (n=15, each 48 hours for 10 d)
[b] Total organic matter
[c] Soluble sugar
[d] Acridine orange direct counts
[e] Incubated at 25℃ for 3 d on the nutrient agar

from of 0.71- 2.42×10^6 cells · ml^{-1} and 0.13-6.27×10^5 colony forming units (cfu) · ml^{-1}, respectively. There were no significant correlation between total bacteria by AODC to water pH, but viable counts of heterotrophic bacteria increased as the pH became lower.

Identification of heterotrophic bacteria

As it was found that acidification affected the physico-chemical factors and viable counts of heterotrophic bacteria, it was thought that the effects accompanied the changes of species composition. Therefore, the isolates from each batch were identified by genus using the data base of the Vitek^R system. The results of identification for 202 isolates showed 12 genera and 22 species: *Providencia* sp., *Bacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Proteus* sp., *Serratia* sp., *Aeromonas* sp., *Corynebacterium* sp., *Actinobacillus* sp., *Vibrio* sp., *Morganella* sp., and *Enterobacter* sp. The changes in species composition according to pH is summarized in Fig. 1. As the incidence of different genera decreased from 12 at pH 7 to 5 at pH 3, the genus diversity decreased as pH became lower.

On the other hand, 64% of the isolates proved to be gram negative, and 36% gram positive bacteria. Comparing the distribution rate between gram negative and gram positive bacteria according to pH, the rate of gram positive bacteria decreased and that of gram negative bacteria increased as pH became lower (Fig. 2).

G+C content

In an analysis on the G+C content of 202 bacterial

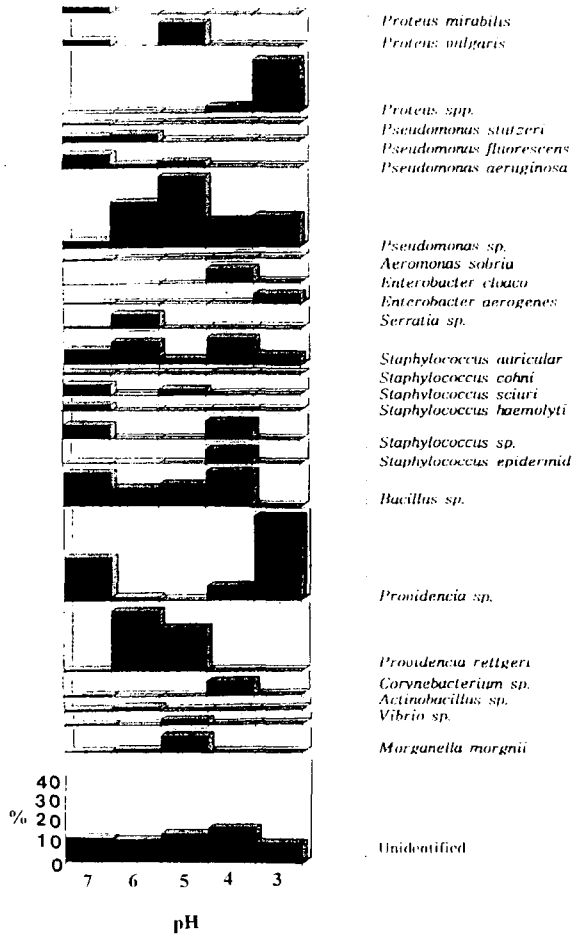

Fig. 1. Summary of the change in size of heterotrophic bacterial population according to pH-gradient. The isolates were identified by the Vitek^R system.

isolates, the range of G+C content was 22.8-77.0 % and that variation became narrow as pH became lower. On the whole, the average G+C content changed little in accordance with pH as the average G+C content of isolate from each batch were 50.5, 55.8, 50.3, 59.6, and 56.5 % at pH 7, 6, 5, 4, and 3, respectively (Fig. 3). In the case of interspecies of some genera such as Providencia and Pseudomonas, however, the average amount of G+C increased as pH became lower (Fig. 4).

Cluster analysis

Numerical taxonomy based on simple matching coefficient showed 24 clusters at the level of 80% similarity (Table 3). The number of clusters of isolates decreased as pH became lower, since incidence of clusters decreased from 22 at pH 7 to 5 at pH 3. The results of cluster analysis were correlated to the pattern of identified isolates on the basis of the Vitek^R system data base (Fig. 1). Also, unknown bacteria for interspecies association on the basis of the physiological test were compared to identified bacteria by the Vitek^R system. Unknown bacterial isolates from pH 7

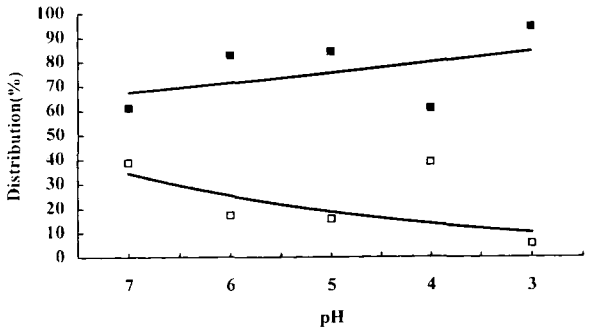

Fig. 2. Comparing the distribution rate between gram negative (■) and gram positive (□) bacteria according to pH gradient. Data points are plotted as mean ±95% confidence intervals. Gram negative bacteria, y=4.46X+0.96; Gram positive bacteria, y=-2.32X+83.82.

and pH 6 seem to be belong to the genus *Providencia*, since they all are fermentative, oxidase positive, nonmotile rods.

Richness, diversity, and evenness of isolates according to pH are shown in Table 4. In general, the diversity index in the acidified environment ranged from 1.0 to 3.0 (Rattray and Logan, 1993). This index can be used as an indicator for various environmental stresses because the diversity index of a microbial community can become lower under certain kinds of environmental stress (Wassel and Mills, 1983). In this study, the diversity index of species was 2.37 at pH 7 and 1.13 at pH 3, hence the diversity index decreased as pH became lower.

In order to establish an appropriate level of similarity for clustering the isolates, a computer program for rarefaction was used to generate plots comparing the cumulative number of clusters encountered. In Fig. 5, the 95% confidence bands for curves at pH 7 and pH 6 greatly overlap indicating that these 2 communities had similar diversities. The majority of these curves is below the confidence band of pH 6, indicating that the 3 assemblages in the batch were less diverse than in the pH 6 and pH 7 samples. The trend in rarefaction curves appeared to be related to the diversity of the microbial communities, so statistical significance of the species diversity of numbers of samples with different

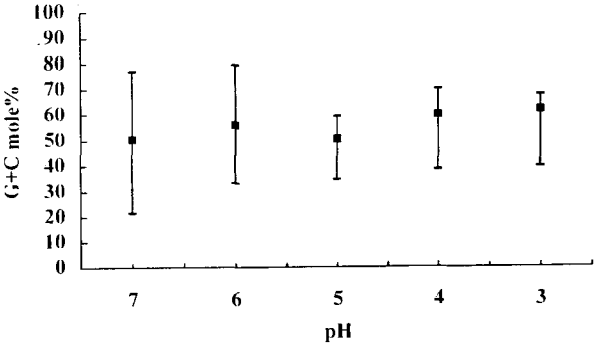

Fig. 3. The range of G+C content ($\bar{x}$, range) according to the pH gradient. G+C content were obtained by high performance liquid chromatography.

Table 3. Distribution of bacteria among the cluster analysis

Sample	No of isolates	No of clusters[a]	Distribution of isolates among clusters[b]
pH 7	71	22	16, 13, 7, 4, 3(2), 2(8), 1(8)
pH 6	56	18	18, 13, 5, 4, 2(2), 1(12)
pH 5	27	12	7(2), 2(3), 1(7)
pH 4	29	8	6, 5(2), 2(2), 1(3)
pH 3	19	5	14, 2, 1(3)

[a]Clusters formed at the 80% level of similarity.
[b]Values represent the number of individuals in a cluster; that is, the size of the cluster (frequency, if >1). Values in parentheses represent the number of clusters of that size.

size according to pH could be demonstrated.

Discussion

For the past 3 yr, we have studied the effects of environmental factors that affect regional and seasonal changes of microbial populations at several reservoirs in industrial areas and big cities by multiple regression analysis (Ahn et al., 1995). Populations of bacteria in small reservoirs with depths of 5 meters or less were greatly influenced by the concentrations of organic and inorganic nutrient from external inflows and by seasonal algal blooms caused by eutrophication. In contrast, the changes in bacterial populations in large lakes were explained by the influence of pH as 40% were recorded in case of total bacteria and 68% in the case of heterotrophic bacteria (Ahn et al., 1995). Therefore, we tried to predict the effects of acidification on the aquatic microbial ecosystem in this area by analyzing the structure of the microbial community in a batch culture system with an artificial pH gradient from pH 7 to 3.

According to Rattray and Logan (1993), pH has little influence on the difference in diversity of heterotrophic bacteria in an acidified area and a non-acidified area. However, it is generally known that microorganisms are so sensitive to acidic stress that their population size, diversity, and activities are reduced (Wassel and Mills, 1983). It has also been discovered that some of them have resistance to environmental stress (Schlindler et al., 1989). According to Dugan et al. (1970), only a

Table 4. Diversity index of heterotrophic microbial communities in batch culture system

Sample	Richness (R2)[a]	Diversity (H')[b]	Evenness (E1)[c]
pH 7	1.90	2.37	0.86
pH 6	1.75	2.33	0.91
pH 5	1.54	1.79	0.86
pH 4	1.33	1.67	0.93
pH 3	1.15	1.13	0.70

[a]Richness index(Menhinick index, R2) : $R2=S/\sqrt{N}$
(S=total number of species, N=total number of individuals)
[b]Diversity index(Shannon index, H') : $H'= -\sum_{i=1}^{S}(p_i \ln p_i)$
($p_i=n_i/N$(n_i : number of individuals of the $_i$th strains)
[c]Evenness index(Pielou index, E1) : $E1=H'/\ln(S)$
(S=total number of species, H'=Shannon index)

few kinds of bacteria such as *Bacillus, Micrococcus, Sarcina, Escherichia, Aerobacter, Thiobacillus, Crenothrix,* and *Microsporium* can live in acidified aquatic ecosystems. In this study, we concluded that the lower the pH, the higher the distribution rate of gram negative bacteria. Under pH 4, we could isolate *Pseudomonas, Providencia, Proteus, Bacillus, Staphylococcus, Corynebacterium,* and *Enterobacter.* These are found easily in freshwater habitats (Fletcher, 1979). Most of them were genera included in the Enterobacteriace family, thus we conclude that it has a close relationship with the inflow of sewage. In an analysis of the distribution rate of dominant genera for each pH level, that of *Providencia* was 21% and 31% at pH 7 and pH 6, respectively, that of *Pseudomonas* was 37% at pH 5, that of *Staphylococcus* and that of *Pseudomonas* was 29% and 14%, respectively at pH 4, and that of *Providencia* was 37% at pH 4 (Fig. 1). On the other hand, the distribution rate of *Serratia* and *Aeromonas* was below 10% at a pH higher than pH 5, and did not appear at pH 4 and pH 3. Thus, it is thought that they are very sensitive to acidic stress. There are some differences in the distribution rate among dominants in accordance with the pH level, however, several genera such as *Providencia, Pseudomonas, Staphylococcus,* and *Bacillus* existed in equal numbers. It is thought that these groups of heterotrophic bacteria are

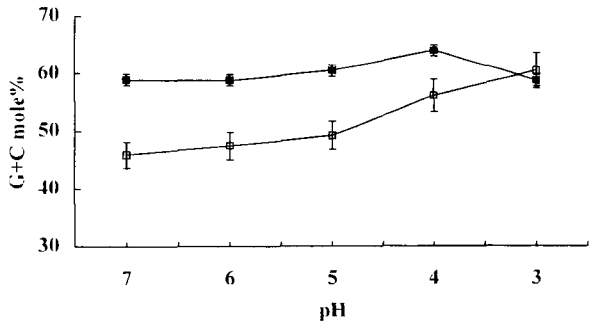

Fig. 4. The average G+C content of interspecies of dominant species according to pH gradient; *Providencia* sp. (□), *Pseudomonas* sp. (■). The G+C contents were calculated from the averages of five measurements. The error bars indicate standard deviations.

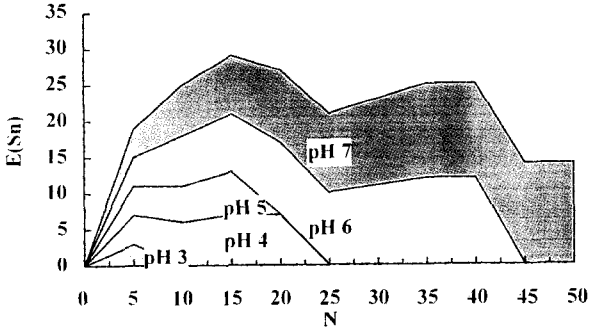

Fig. 5. Rarefaction curves comparing diversity of the 5 communities according to pH gradient. E(Sn) is the expected number of guilds present in a subsample of N individuals drawn from total samples. The curves represent the diversity of the community at each pH.

well-habituated major genera that contribute to nutrient cycles in the process of acidified environment of this research area.

In an analysis on the amount of G+C of isolates, the variation range of G+C content became narrow as the pH decreased. The average amount of G+C was relatively large below pH 4, but the overall change was little in accordance with the pH. However, analyzing the average amount of G+C at each pH level for the genus *Providencia* and *Pseudomonas* that appeared evenly at all levels, we could find a clear relationship between pH and G+C content. The G+C content range of *Providencia* was 39%-60%, and that of *Pseudomonas* was 59%-71% (Fig. 4). The average amount of G+C increased significantly as the pH decreased. We even observed that the same genus can have a different G+C content depending on acidic stress, and the G+C content of bacteria increases incrementally with environmental stress.

On the basis of several years' field study of a certain ecosystem, we analyzed the effect of acidification on the structure of a heterotrophic bacteria community by using an artificial pH gradient batch culture system (Ahn et al., 1995). Until now, serious damage by acidification has not been reported in Korea. It is expected that this study will be helpful in predicting future structural changes in Korean freshwater heterotrophic bacterial communities in the process of acidification. Results of this study can serve as a basis for future studies of various phenomena and functions of the microbial ecosystem in the process of acidification.

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