Spatial Distribution of the Microbial Community to Develop on a Slide Glass Submerged in River Water

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The spatial distribution of a microbial community in an oligotrophic region of the Tamagawa River which developed on the surface of a slide glass submerged in the river was observed by microscopy along with the course of biofilm formation. Bacterial cells attached to the slide glass after one hour and increased in number on the 4th day of submergence. Algal cells began to adhere to the bacterial cells on the 8th day, increasing the thickness of the biofilm. However, the surface of the slide glass was not entirely covered with bacterial cells. In the process of biofilm formation each of the biofilm microbes was sketched and enumerated. The results showed that bacterial cells pioneered the colonization and made up the base of the biofilm. The frequency and abundance of bacterial cells in each square implied bacterial cell division on the slide glass.

Key words: biofilm forming process, bacterial community, pioneer, spatial distribution, algae

The epilithon microbial community that develops on pebbles in riverbeds plays an important role in the degradation of allochthonous or autochthonous substances in the river ecosystem. The epilithon is composed of bacteria, algae, protozoa and larvae of large organisms. When the epilithon is washed away, for example in a large flood, it gradually redevelops on the riverbed, responding to the river flow and water quality⁹⁾. It was reported that in the process of biofilm formation, bacterial cells were the first to attach to the substrate. They then excreted substances to which algal cells adhered and increased in number, consequently forming the epilithon^{1,10)}.

The objective of this study was to identify the pioneer microbe of biofilm formation, and clarify how they create the biofilm on the substrate. To assess the bacterial activity to divide on the substrata, bacterial counts were made both in colony forming units and directly using acridine orange. The early process

of the formation on artificial substrata submerged in river water was observed by epifluorescence microscopy, and the area occupied by each component of the microbial community was estimated based on the grids of microscopic fields.

Materials and Methods

Study site

The study site was the middle stream at Hamura-weir in the Tamagawa River flowing through Tokyo, Japan. Hamura-weir is situated at the upper point of the middle region, 70 km from the source of the river. The flowing water surface was broad and there was no shade. The study was carried out from the 14th of December 1997 to the 26th of January 1998. Water quality at Hamura-weir was oligotrophic and the concentration of dissolved organic carbon (DOC) was around 1 mg C l⁻¹ throughout the year¹³⁾. Water velocity at the study site was 30~40 cm sec⁻¹ year-round, and water temperature at the time of the study

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was 3.1°C to 9.0°C.

Artificial substrata

A slide glass (Matsunami, thickness 1.2~1.5 mm, 76 × 26 mm) was chosen for the artificial substrata to observe the adherent microorganisms, because it was transparent and cells attaching to the surface of the substrata could be viewed by microscope⁷⁾. New slide glasses were used without washing. They were checked for microbial contamination under the microscope. The glasses were fixed with double-sided adhesive tape to an iron angle, without space between them to avoid any distortion of the flow. The iron angle was submerged in the river water on December 14. The water depth above the upper surface of the slide glass was 10~15 cm and the glasses were placed parallel to the water flow.

Sampling procedure

Six submerged slide glasses were taken from the river water carefully so as not to disturb the biofilm on the glass surface and transferred to a sterilized polyethylene bottle filled with river water at each sampling time. Three of them were examined to estimate biomass and the others were employed for observation under the microscope. All bottles were maintained at 5~7°C for about 2 hours until the following procedure was carried out. Samplings were conducted one hour after submergence (0 day) and on the 4th, 8th, 12th, 17th, 21st, 25th, 31st and 43rd days after submergence.

To measure dry weight, ignition loss, chlorophyll contents, colony forming units (CFU) of bacteria and acridine orange direct counts (AODC), the slide glass was taken carefully from the polyethylene bottle and rinsed gently with sterilized tap water several times. A newly unsealed micro cover glass (Matsunami, 24 mm × 50 mm, thickness No. 1) was rinsed with sterilized tap water and placed on the biofilm to define the observation area. The material around the micro cover glass was wiped with paper (Kimwipe Wipers S-200), and the surface of the slide glass was again rinsed carefully with sterilized tap water. The material in the 24 × 50 mm area of the biofilm on the slide glass was scrubbed with the edge of the new micro cover slide,

and collected with a suitable volume of sterilized tap water then put into a new sterilized polyethylene bottle. Since residual material on the slide glass was not detected by microscopic observation (× 100), the efficiency of scrubbing was to be regarded as high enough to measure the biomass of the biofilm that developed on the slide glass.

Estimation of the biofilm biomass

A 100-ml volume of the collected suspension of biofilm from the slide glass was dispersed aseptically by sonication at 5~6°C for 60 sec (Ohtake No. 5202-T, 20 kc, 50 W). These conditions gave the largest number of CFU in a preliminary test. Subsamples of sonicated biofilm suspension were employed for the measurement of dry weight, ignition loss and chlorophyll content by methods described elsewhere⁹⁾.

CFU of the bacterial community from the sonicated biofilm suspension were enumerated as described previously⁹⁾. Aliquots (0.1 ml) of successive dilutions (10^{-1}) of the sonicated subsample were spread in triplicate on the surface of a 1/8 PYG agar plate. This medium gave the largest number of colonies in a preliminary test. The 1/8 PYG agar contained 0.25 g of polypepton (Kyokuto Co.), 0.13 g of yeast extract (Difco), 0.07 g of glucose, and 15 g of agar powder (Kyokuto Co.) in one liter of tap water, pH 7.0. All plates inoculated were incubated at 20°C for 28 days.

AODC of the biofilm on the slide glass was determined by partially modified acridine orange epifluorescence microscopy as described by Hobbie et al⁶⁾.

Direct observation of microorganisms

The submerged slide glass was taken from the river-bed and rinsed off with 0.1 mM phosphate buffer gently. The surplus phosphate buffer was adsorbed by filter paper and 0.1% of acridine orange (Merck) solution was poured directly on to the slide glass, and left to stain for 30 minutes. The slide glass was rinsed of excess AO with 0.1 mM phosphate buffer and air dried, then mounted with immersion oil (Nikon FL) and observed with an epifluorescence microscope (15 × 100) with a SWB (Super Wide Blue excitation filter)

cube (Olympus BX-60).

A micrometer with 20×20 squares was set in the eyepiece lens and a grid of 400 squares ($10,000 \mu\text{m}^2$) was used as a unit throughout the experiments. To classify the microorganisms, the microbial community was grouped into four categories based on the members; (A): algal cells consisting mostly of diatoms, (B): bacteria existing as a single cell, (C): filamentous Cyanobacteria, and (D): debris which were not grouped into any of these categories and non-living things.

On the assumption that a microscopic field consisting of 400 squares was one quadrat, the distribution of the microbial community on the slide glass was observed by epifluorescence microscopy. The bacterial and algal cells observed in 400 squares were precisely sketched one by one on sectioned paper, and the number of squares occupied by each category of the microbial community was counted disregarding overlapping squares. The total number of squares counted exceeded 400. The area occupied by each category was shown as the number of squares, and described as the percent coverage for 400 squares. Twenty microscopic fields were counted for one sample. Algal cell counts were carried out based on the sketch of all unit squares of the microscopic field. Algal cell counts were expressed as the total number of algal cells and were not classified.

The frequency of bacterial cells in one square of a grid was calculated from the sketch of all 400 squares. Bacterial cells of the epilithon were very small and several cells were seen in one square. The frequency of these cells was described based on the number observed in one square. Frequency 1 indicates one microorganism in a square, frequency 2 means two microorganisms in one square and so on. Frequency 5 indicates over five microorganisms in one square. The abundance of bacterial cells in 400 squares was thus assessed and expressed along a time course.

Results and Discussion

Increase in biofilm biomass on the submerged slide glass

The increase in dry weight, ignition loss, chloro-

phyll content, CFU and AODC are shown in Fig. 1. A remarkable increase in the biomass of the biofilm was found on the 21st day of the experiment and the biofilm did not exfoliate until the 43rd day, the last day. Early in the submergence, however, the biomass of microorganisms, ignition loss (Fig. 1a), and chlorophyll content (Fig. 1b) were too low to detect by the methods employed. In contrast to the slow development of biofilm during the first 20 days, microorganisms did attach to the slide glass and rapidly proliferated during this period as is shown in Fig. 1c. Numbers of bacteria determined both by CFU and AODC were almost maximal on the 4th day of the

Fig. 1. Increase of dry weight (○), ignition loss (△) (Fig. 1a), chlorophyll a (◇), phaeophytin a (□) (Fig. 1b), colony forming unit of bacteria (CFU) (○) and acridine orange direct count (AODC) (●) (Fig. 1c) in biofilm developed on the slide glasses submerged in river water at Hamura in the Tamagawa River from 14th Dec. 1997 to 26th Jan. 1998. Bars indicate SD.

experiment. Hendricks⁵⁾ reported that bacterial sorption to slide glass in a continuous culture of river water occurred just one day after incubation. The result obtained in this study agreed with that report.

Abundance of the cells of each component of the biofilm

The increase in the number of cells of each component of the biofilm community on the slide glass

Fig. 2. Increase in the number of cells of each component of the biofilm community developed on slide glasses soon after submerging in river water. ■: Bacteria, ♦: Algae, ▲: Debris. Bars indicate SD.

Fig. 3. Percent coverage of each component of the biofilm developed on slide glasses soon after submerging in river water. ■: Bacteria, ♦: Algae, ●: Filamentous Cyanobacteria, ▲: Debris. Bars indicate SD.

submerged in the river water is shown in Fig. 2. A single bacterial cell had attached to the slide glass one hour after submergence, and the number of single bacteria increased rapidly after the 4th day. A difference in the bacterial count enumerated by CFU and AODC was observed on the 4th day from the suspension of scrubbed biofilm. This suggests that the bacteria directly detected on the glass were effectively

recovered by the cultivation method employed. On the other hand, the bacterial number on the 21st day declined, probably due to concealment by algae. Increases in the number of algal cells were delayed by bacterial proliferation. Debris was recognized independently of the growth of bacteria and algae from the 12th day of the experiment.

Fig. 4. Microscopic photos of biofilm developed on the slide glass at one hour after submergence (A), 4th day (B), 8th day (C), 12th day (D), 17th day (E) and 21st day (F). Bar indicates 20 μ m.

Changes in the coverage of each component of the biofilm

The coverage of each component of the biofilm on the slide glasses was evaluated as a percentage (see Fig. 3). Individual bacterial cells occupied a large portion of the grid field (35%) and were the dominant flora on this artificial substrata from the beginning to the 17th day of submergence. The occupied area, however had not increased over 42% on the 21st day. Filamentous Cyanobacteria occupied fewer than 12% of the field. In contrast, the area occupied by algal cells increased rapidly between the 17th and the 21st day, and occupied 75% of the slide glass. Algae might be dominant in this period on the submerged substrata (Fig. 3). The results showed that bacteria were the first to colonize the slide glass in the river water and then algal cells attached and delayed the bacterial invasion. The first bacteria to attach may excrete polymer as mentioned previously^{2,3,8)}.

Microscopic photos of the slide glass one hour after submergence, and on the 4th, 8th, 12th, 17th and 21st days of submergence are shown in Fig. 4. The photo taken on the 21st day showed that the biofilm was growing vertically and the focuses of bacteria and algae could not adjust on the same plain. These algal cells increased vertically before the surfaces of the slide

glasses were filled with bacteria. The surface of the newly submerged substrata was not covered entirely with the microorganisms.

Changes in frequency of bacterial cells

The frequency and abundance of the bacterial cells in one microscopic field early in this experiment are shown in Fig. 5. A square of frequency 1 was recognized at one hour after submergence and the number of squares of frequency 1 increased on the 4th day. These individual bacterial cells might have a high adsorption force for surfaces such as slide glass as described by Morisaki et al¹¹⁾. Fletcher⁴⁾ showed the culture conditions for bacterial attachment to polystyrene and clarified the relationships between attached cells and substrata. Nagata and Watanabe¹²⁾ observed the pattern of colonization in a slide culture of a bacterial community in river water. The number of squares of frequency 1 was maximal on the 8th day, but then decreased gradually. The coverage of frequency 1 did not exceed 20% of the grid field. The abundance of frequency 2, recognized on the 8th day, was later than that of frequency 1, and was maximal on the 12th day. The abundance of frequency 3 reached a maximum on the 17th day. The abundance of frequencies 4 and 5 of bacterial cells showed a peak on the 12th and 17th day, respective-

Fig. 5. Frequency and abundance of bacterial cells in each square of one microscopic field developed on slide glasses soon after submerging in river water. (Frequency 1, 2, 3 indicate that one, two, three microorganisms existed on a unit square.)

ly. As the frequency increased, the abundance moved to a later date. In spite that virgin squares remained on the slide glasses, the abundance of frequency 1 decreased on the 12th day. This suggested that viable bacteria had proliferated on the slide glass and frequency 1 changed to frequency 2 or 3. These results also suggested that flowing bacteria could proliferate in river water by attaching to newly contacted substrata as early as eight days after submergence in cold water. After eight days of submergence, the attached bacteria increased in number. Algal cells proliferated after bacterial cell growth. Cold water suppressed the bacterial growth and consequently, the bacterial distribution could be closely observed.

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