Hydrobiologia (2005) 539:27–34
DOI 10.1007/s10750-004-3082-5

Primary Research Paper

Bacteria biofilm encourages algal immigration onto substrata in lotic systems

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Received 9 May 2004; in revised form 14 July 2004; accepted 8 September 2004

Key words: algae, algal immigration, bacteria, periphyton, succession

Abstract

I tested the effect of the density of attached bacteria on the amount of algal immigration in the early development of a periphyton community in an artificial stream by manipulating the density of the attached bacteria. Three densities were prepared by regulation of the incubation time. A suspension of algae was added to the stream, and the degree of algal attachment to substrata was compared among the treatments. Algal immigration was proportional to the density of attached bacteria on all substrata (glass, PVC, and slate), although density differed among substrata. Analysis of covariance (dependent variable, amount of attached algae; covariate, bacterial density) showed significant relationship between amounts of attached algae and bacterial densities, but did not show significant differences in the slopes and adjusted means among substrata. When acrylic beads were added with the suspension of attached algae, significant linear correlation was obtained between the amount of attached algae and the amount of acrylic beads on the substrata. Algal immigration was due to non-selective adsorption by attached bacterial biofilms on substrata, although the extent of bacterial colonization and biofilm formation may be affected by the substrata and other environmental factors (e.g., current conditions and water temperature).

Introduction

Periphyton communities consist of bacteria, fungi, algae, and protozoa, and grow on stones and cobbles. Algae are the primary producers in river periphyton communities, and have an important role in the development of the community and as the base of the food web in river ecosystems. The development of heterotrophic organisms in the periphyton community generally depends on algal production (Haack & McFeters, 1982; Romani & Sabater, 1999). Attached bacteria are important decomposers of both algal products and external organic matter input (leaf litter and dissolved organic matter derived from catchment and canopy) (Findlay & Howe, 1993).

On the other hand, many researchers have observed bacterial attachment and growth on substrata before algal attachment in lotic and lentic systems (e.g., Aizaki, 1980; Hudon & Bouget, 1981; Hoagland, et al., 1982; Liu et al., 1993; Ács et al., 2000; Morikawa & Shibuya, 2000). This situation suggests that the attached bacterial biofilm and bacterial mucilage have an important role in the enhancement of algal attachment to the substrata. However, the roles of attached bacteria in the process of algal immigration are not clearly understood. Because the variety of environmental factors (e.g., current velocity, water temperature, suspended algal density in flowing water, and kind of substratum) may also affect algal immigration, the amount and duration of algal immigration have not been fully clarified by previous studies.

A major factor that has confused the role of attached bacteria in algal immigration is the trophic interaction between algae (autotrophs) and

bacteria (heterotrophs). If algae were observed with attached bacterial biofilm, it can also be interpreted that bacteria feed on the organic matter excreted by the algae and increase around the algae. This makes it difficult to pin down the colonization sequence and the importance of attached bacteria in secondary algal immigration. Therefore, to understand the role of attached bacteria in algal immigration, experimental analysis designed to exclude the effect of trophic interactions is needed.

The objective of the present study, was to clarify the role of attached bacteria in the process of algal immigration. The effect of attached bacterial cell density on the amount of attached algae was tested in three experiments. The process of algal attachment on substrata and the significance of bacterial colonization in periphyton succession are discussed.

Methods

Experimental apparatus

Experiments were conducted in an outdoor artificial stream at Hachioji, Tokyo,and Japan (35° 37′N, 139° 23′N), from May to October 1999. River water (120 l) was circulated in eight straight stainless steel flumes (1.2 m long, 10 cm wide). The flow through each flume was 8–9 l min^{-1}, which resulted in a water velocity and depth of approximately 30 cm s^{-1} and 2 cm, respectively. The experimental water was kept at a constant temperature (22 ± 3 °C) with coolers and heaters. Water used for the experiments was collected from the Tama River at Hamura (35° 43′N, 139° 20′N) in southwest Tokyo. The Tama River flows across the urban area of the Kanto region, and thus the concentrations of dissolved inorganic phosphorus (DIP) and dissolved inorganic nitrogen (DIN) during the experiments were high: 19–50 and 400–1200 μg l^{-1}, respectively. On the other hand, Chlorophyll-*a* (chl-*a*) during the experiments was relatively low (0.8–3 μg l^{-1}).

Experimental designs

To test the role of bacterial colonization in the process of algal attachment to substrata, the bacterial cell densities on the substrata were regulated, and the amounts of attached algae were measured. The river water was filtered through a Whatman GF/F filter, and the filtrate, containing bacteria but not algae, was circulated in the artificial stream apparatus. To achieve different attached bacterial cell densities on the substrata, artificial substrata were put in each flume on three different dates. Antecedent experiments showed that attached bacterial cell densities on day 1 after the onset of experiment were $2–6 \times 10^4$ cells cm^{-2} (Hodoki, submitted). The attached bacterial cell densities increased exponentially until after 6 days and reached a density of $1–4 \times 10^6$ cells cm^{-2}, though the net growth rate, thereafter, became slower (Hodoki, submitted). On this basis, artificial substrata in the present study were introduced on days 0, 3, and 6 after the onset of experiment. On day 6, a suspension of attached algae previously collected from the Tama River periphyton community was added to the experimental stream at a final concentration of 2–3 μg chl-*a* l^{-1}. On day 8 (2 days after the algal addition), the amounts of attached algae on substrata were measured. Three experiments were performed. To determine the relationship between the bacterial cell density and the amount of attached algae, precombusted (420 °C, 3 h) frosted glass was used as the substratum (Exp. 1). To assess whether the substratum material directly affects the amount of algal immigration, frosted glass tiles, PVC tiles (roughened on the top), and slate tiles were used as substrata, and the bacterial cell density and the amounts of algae on the substrata were compared (Exp. 2). To clarify whether the bacterial biofilm selectively attaches only algae, a mixture of acrylic beads (20–30 μm in diameter) and algal suspension (final concentration, 2.5 μg chl-*a* l^{-1}) was added to the water, and the relationship between the amount of attached algae and beads on the substrata was analyzed (Exp. 3). This experiment used precombusted frosted glass tiles which were put on the flume bottom every day until day 8. The acrylic beads (Sephacryl, Pharmacia Co., final concentration 7.5×10^2 ml^{-1}, filtered through 30-μm mesh size plankton net) and algal suspension were added on day 8, and the glass tiles were sampled on day 10. All three experiments were done under dark conditions (all flumes were covered with black vinyl films) to avoid the growth of algae once attached on the substrata, and used a block design (all treatments were located in each flume) in four

replications (four flumes). Bacterial cell densities in the water were also monitored and were almost constant throughout the experiments ($0.9–1.3 \times 10^6$ cells ml^{-1}). The density before and after the addition of algal suspension did not significantly change (only $0.5–1.0 \times 10^5$ cells ml^{-1} increased).

Enumeration of algae and bacteria

Ten tiles (3 cm × 3 cm) were placed on the bottom of each flume as substrata on each date, and five randomly selected tiles were removed at each sampling date. Only the periphyton on the top of each tile was evaluated. That on the back and sides of the tiles was wiped off with Kimwipes (Kimberly–Clark), and the tile was rinsed with Milli-Q water. Tiles were then transferred to 100 ml Milli-Q water, and periphyton was removed by gentle sonication (20 kHz, 50 W, 3–4 min) according to Claret (1998). Aliquots of the suspension were fixed with glutaraldehyde (final concentration 1%) for enumeration of attached bacteria and with sodium azide (final concentration 3%) for enumeration of attached acrylic beads. The remaining suspension was filtered through a precombusted Whatman GF/F filter, and the filters were used for analysis of chl-*a*. After extraction with 100% methanol for 24 h, Chl-*a* concentrations were determined by fluorometer (TD-700, Turner Designs). Bacteria were stained with 4′,6-diamidino-2-phenylindole (DAPI, final concentration 10 μg ml^{-1}) for 10 min at room temperature and filtered through a black Nucleopore filter (pore size 0.2 μm). The filter was washed with Milli-Q water and mounted with non-fluorescent immersion oil (Olympus) on a glass slide. Bacterial cells were counted at 1500 × magnification under an epifluorescence microscope (Olympus, model IMT-2 RFM) equipped with a mercury lamp (100 W). The excitation wavelength was in the UV range. At least 300 cells in total were counted in eight optical fields. After concentration by sedimentation, acrylic beads were counted in a Fuchs–Rosenthal hemacytometer or a plankton counting chamber (1 ml volume).

Statistical analysis

Before statistical analysis, all data were log-transformed to reduce non-normality and heteroscedasticity (Sokal & Rohlf, 1997). One-way factorial ANOVA was used in Exp. 1 to test the effect of bacterial cell density on the amount of attached algae (Sokal & Rohlf, 1997). Analysis of covariance (ANCOVA) was used in Exp. 2 (Sokal & Rohlf, 1995). The independent variable was the amount of algal immigration; the covariate was bacterial cell density on the substrata. The significance of the slope in pooled regression and difference among the separate slopes were tested first, and then the difference in adjusted means among treatments was tested. A significant difference in the slope means that there is an effect of attached bacterial cell density on the amount of algal immigration. Differences among slopes imply differences in the effect of attached bacterial cell density on the amount of algal immigration due to substrata. A difference in the adjusted mean implies differences in the attachment of algae independent of the existence of attached bacteria. A significant difference in the adjusted means among treatments (substratum materials) would be the evidence that the kind of substratum directly affects algal immigration. In Exp. 3, regression analysis was used to show the relationship between the amounts of attached algae and acrylic beads.

Results

Figure 1 shows the effect of attached bacterial cell density on the amount of attached algae. A zero value of the attached algal density could not be obtained, owing to contamination by unknown fluorescent matter during sample treatments (Fig. 1a). The amount of chl-*a* on the washed and sterilized new glass tiles was determined in the low bacterial cell density condition before addition of algae. Before addition of the algal suspension on day 6, the amounts of chl-*a* did not significantly differ among the three treatments (Table 1). Therefore, there were no significant effects of the contamination on the amounts of attached algae before the addition of the algal suspension. On the other hand, the attached bacterial cell densities differed between high and medium bacterial cell densities (high 1.1×10^6 cells cm^{-2}, medium 4.5×10^5 cells cm^{-2}) and were successfully controlled into three grades (Fig. 1b). On day 8 (2 days after addition of algal suspension), both

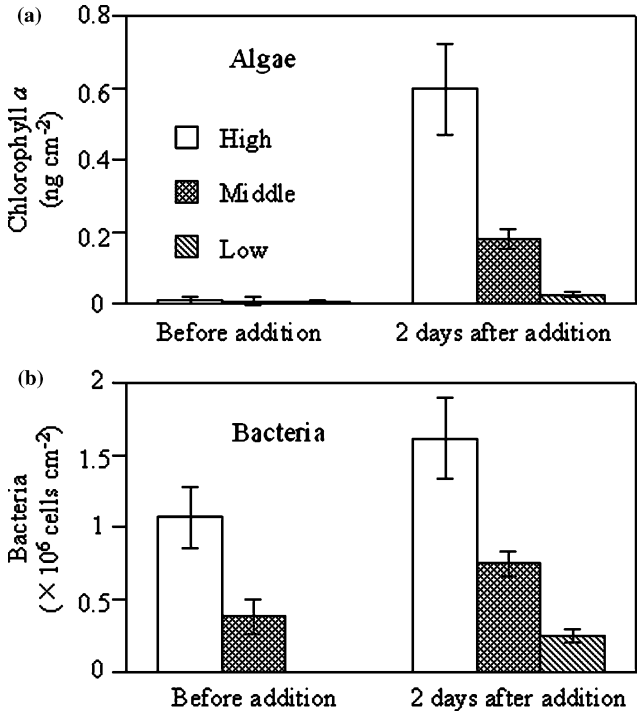

Figure 1. Amounts of attached algae (a) and attached bacterial cell densities (b) on substrata before and after addition of suspended algae. Bars indicate means ± 1 SD.

Table 1. Result of ANOVA of the amounts of attached algae before and after addition, and bacteria density

Analysis	Source of Variation	df	MS	*F*	*p*
Chlorophyll *a*	Treatments	2	0.205	1.54	ns
(Before addition)	error	9	0.133		
Chlorophyll *a*	Treatments	2	1.912	175.86	< 0.001
(After addition)	error	9	0.011		
Attached	Treatments	2	0.665	140.06	< 0.001
bacteria	error	9	0.005		

the densities of attached bacteria and the concentrations of chl-*a* were significantly different between treatments (Table 1), and the amounts of chl-*a* responded positively to the increasing bacterial cell densities (Fig. 1).

The experiment using three different substrata also showed positive relationships between the densities of attached bacteria and chl-*a* (Fig. 2). The bacterial cell densities were different on each material, decreasing from PVC to slate to frosted glass (Fig. 2b). However, treatments with the same bacterial cell densities showed almost the same amount of chl-*a*, irrespective of substratum (Fig. 2). Although the slope of pooled regression significantly differed from 0 (df = 1,30, $F = 524.3$, $p < 0.001$) and differences among slopes were not significant (df = 2,30, $F = 2.8$, $p > 0.05$), the result of ANCOVA did not show significant differences in adjusted means among treatments (df = 2, 32, $F = 1.90$, $p > 0.2$).

Figure 3 shows the results of the attachment of acrylic beads. There was a strong significant correlation between the amounts of beads and chl-*a* ($n = 32$, $r^2 = 0.84$, $P < 0.001$). Thus, the attached bacterial biofilm could also trap other suspended particles in flowing water.

Discussion

Is the biofilm a prerequisite for algal immigration (Hoagland et al., 1982; Korte & Blinn, 1983; Morikawa & Shibuya, 2000)? Most previous studies used only microscopic observation, and the periods of observation of algal attachment were

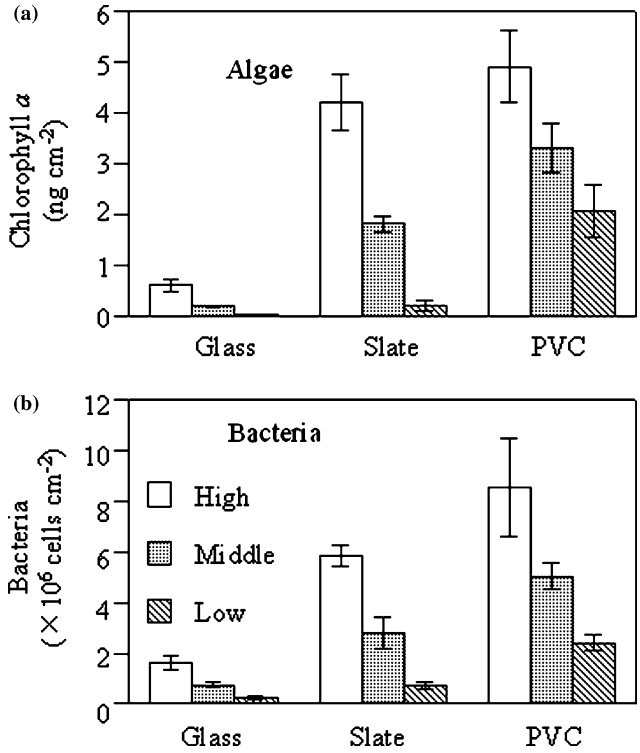

Figure 2. Amounts of attached algae (a) and attached bacterial cell densities (b) on different substrata after addition of suspended algae. Bars indicate means ± 1 SD.

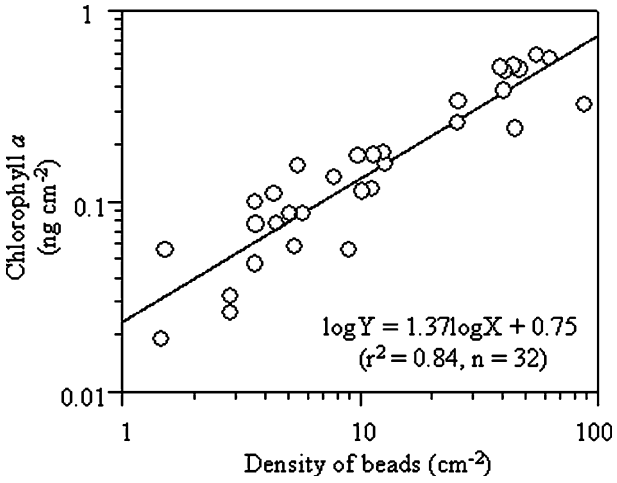

Figure 3. Relationship between the amounts of beads and attached algae on substrata.

not constant (ranging from an hour to a few weeks); thus, the role of attached bacteria in algal immigration could not be generalized. The present results make it clear that the amount of attached algae increased significantly with increasing attached bacterial cell density on all substrata (Fig. 2). This finding clearly demonstrates the importance of the biofilm in the process of algal immigration. Moreover, algal immigration independent of bacterial adsorption seems to be minor process, because differences in the substrata did not directly affect the amount of algal immigration

(Fig. 2), and a strong linear correlation between the amounts of beads and chl-*a* was observed (Fig. 3).

The results of the previous and present studies suggest the following. (1) Periphytic algae suspended in flowing water may have a relatively low potential for adherence (McIntire, 1966; Reisen & Spencer, 1970). (2) Bacteria, which can attach themselves to substrata more rapidly than can algae, grow, and form a biofilm (e.g., Liu et al., 1993). (3) The bacterial biofilm adsorbs or traps the suspended matter in flowing water, including suspended algae (present study). (4) Algae directly attach to the solid surface (Daniel et al., 1987).

In this process of algal immigration, one of the most important features is the adsorption of suspended algae by the bacterial biofilm. It is generally accepted that the periphyton community traps algal cells by its architecture and excreted mucilaginous compounds (Hoagland et al., 1982). However, the trapping ability of the biofilm has not been clarified. Liu et al. (1993) reported that the biofilms on artificial substrata consisted mainly of bacterial excreted carbohydrates (mucilaginous matter) in the early development of the periphyton community in an artificial stream and marine littoral zone. DiSalvo & Daniels (1975) experimentally demonstrated that a marine attached bacterial biofilm promoted the settlement of suspended particles (polystyrene latex, 0.8 μm diameter) on substrata. In the present study, there was a strong correlation between the amounts of acrylic beads and suspended algae trapped on the substrata (Fig. 3). Therefore, the non-selective entrapment of suspended matter by attached bacteria appears to be the major cause of early algal immigration.

Another important feature is the length of the lag period until the attached algae are detected. Aizaki (1980) reported that attached bacteria grew on substrata before algal attachment and showed that there was a negative linear relationship between water temperature and the lag period of algal attachment on substrata in an *in situ* experiment in the Tama River. Because water temperature affects the growth of attached bacteria, this result shows that the development of attached bacteria regulates algal immigration.

On the other hand, Stevenson (1983) reported no lag period and diatom immigration rates of 30–1500 cells cm^{-2} day^{-1} in Fleming Creek. Stevenson experimentally demonstrated that water current velocity and microturbulence near the substratum surface affect the rate of immigration of diatoms onto the substratum, and that adsorptive characteristics of the substratum play a minor role in algal immigration. However, Korte & Blinn (1983) reported, from scanning electron microscope observations, a rapid formation of a bacterial biofilm (within 2 h) before algal attachment, and algal attachments, thereafter, within a day. Ács et al. (2000) also observed a rapid colonization of bacteria (within 3 h) followed by development of *Diatom vulgaris* (within 6 h). Therefore, in these cases, it can be considered that the initial bacterial biofilm was formed not only by bacterial growth but also by accumulation on the substrata owing to high immigration rates of suspended bacteria in flowing water. Probably, as Stevenson (1983) demonstrated, flow conditions near the substratum surface control the rate of impingement of suspended particles on substrata and influence algal immigration. Therefore, the existence of a bacterial biofilm in the process of algal attachment cannot be denied.

Although a positive relationship was found between the amount of attached algae and bacterial cell density on all substrata, the increase rate of attached bacteria differed among substrata (Fig. 2). Because of the necessity of mutual comparison among studies, there are arguments over which material is the best for analyzing periphyton community structure (Lamberti & Resh, 1985; Cattaneo & Amireault, 1992). Because physical characteristics of substrata differ among materials (e.g., surface microstructure, surface tension, and electric charge), the kind of material affects the process of bacterial attachment (Dexter et al., 1975). For example, Fletcher & Loeb (1976) found highest colonization rates in marine bacteria on substrata with surface tensions between 19 and 43 dynes cm^{-1}. The nature of materials also affect species composition of initial colonizing attached bacteria in the coastal environment (Dang & Lovell, 2000). In the present study, I mainly used glass substrate because of its easy handling properties for sterilization and combustion. It is not clear what type of quality differences of substrata (e.g., surface tension, charge, roughness, or actual surface area of substrata) results in the cell density differences of attached bacteria. Nevertheless, our

results suggest that these factors affect the density of attached bacteria but not directly the amount of algal immigration (Fig. 2). The nature of materials may influence both the lag time and the amount of algal immigration through affecting the density of attached bacteria.

In the present study, to analyze the relationship between the amount of attached algae and bacteria in the initial development of periphyton community, the trophic interactions between algae (autotrophs) and bacteria (heterotrophs) in periphyton communities were simplified or eliminated by manipulating only the attached bacterial cell density. Although the real interactions and succession in natural periphyton community are much more complex (e.g., Ács et al., 2000; Dang & Lovell, 2000; Asaeda & Son, 2001; Ács et al., 2003), the results of the present study may contribute to clarify the roles of attached bacteria played in the early algal immigration process during the periphyton community development. Previous studies have suggested that periphyton community succession is similar to terrestrial plant succession, which changes from low to high physical structures (Hoagland et al., 1982). Periphyton communities increase by accumulating suspended particles on their high physical structures and excreted mucilaginous matter as they develop (Hoagland et al., 1982). As demonstrated in the present study, the development of periphyton communities is initiated by the formation of the mainly heterotrophic biofilm, and the bacterial biofilm has an important role in the initial colonization by algae in lotic systems.

Acknowledgments

I thank Dr Yasunori Watanabe (Rissho University) for discussion and research support, K. Ohbayashi (University of Tokyo) for discussion and comments on the manuscript.

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