Note

Direct Observation of Bacterial Attachment to Immersed Glass Slide under Epifluorescence Microscopy

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Abstract

Bacterial attachment to the solid surface in the littoral zone of a eutrophic lake was studied by a simple method using a glass slide under epifluorescence microscopy with acridine orange staining. The initial rate of bacterial attachment was around 1×10^5 cell $\cdot$ cm $^{-2}$ $\cdot$ hr $^{-1}$, and the attached bacterial number amounted to about 10^6 cells $\cdot$ cm $^{-2}$ in 24 hr during experiments in June in Lake Suwa. The contribution of attached bacteria with large cell size and that of dividing cells to the total attached bacteria showed a tendency to increase with immersion time.

Key words : bacteria, attachment, epifluorescence microscopy.

Although free-living bacteria are usually the predominant form of planktonic bacteria in aquatic environments, adhesion of bacteria to the solid surfaces is believed to be an important step for the degradation of organic particles including macrophytes (KATO, 1985). Solid surfaces are supposed to be favorite sites for the growth of bacteria, considering the advantage of attachment to nutrient rich surfaces from diluted bulk waters (BREZNAK *et al.*, 1984). The function of attached bacteria in decomposition and nutrient recycling may be emphasized especially in the littoral zone, where various surfaces of suspended particles, macrophytes and sediment can be found. The present study represents an attempt to estimate the planktonic bacterial attachment to the glass slide submerged in lakewater in order to evaluate microbial function in the matter cycle of the littoral zone.

Epifluorescence microscopy has seen widespread use in aquatic microbial ecology over the past ten years as a direct method for bacterial counting. However, it has mostly been applied to planktonic bacteria which are trapped on polycarbonate filters (ZIMMERMANN and MEYER-REIL, 1974; HOBIE *et al.*, 1977). We just applied this method with acridine orange staining to the observation of bacterial attachment to the glass slide with some modifications. The method which we employed is much more simple and direct compared with the former techniques of FLETCHER and LOEB (1979) or PAUL

and LOEB (1983).

In the procedure employed, glass slides were washed by soaking in a 10% nitric acid solution (PORTER and FEIG, 1980) for a few hours and rinsed with particle-free distilled water (PFDW, filtrate of a 0.2 μ m-pore size Nuclepore filter). Then the glass slides held with clips were immersed in *in situ* lakewater. After exposure to lakewater, the slides were removed and air dried. The slides were then immersed in 0.1% acridine orange solution for a few minutes. Surplus acridine orange was washed twice with PFDW and then air dried. A part of the sample on the glass slide was covered with a cover glass using immersion oil. Bacteria attached to the surface of glass slide were counted at $\times 1,250$ with an epifluorescence microscope (Olympus BHS-RFK) equipped with a dry objective of $\times 100$. For each slide at least 20 fields of 42×42 μ m 2 or more than several hundreds of bacteria were counted. The frequency of dividing cells (FDC, HAGSTRÖM *et al.*, 1979) was determined during the counting and bacterial cell size was measured using a micrometer set on the eyepiece.

The employed air drying fixation can be a source of possible errors in the estimation of attached bacterial number, because it took up to 30 min until the sample had fully dried. Thus, we checked whether this process affects bacterial growth, comparing the employed technique with oven drying and formalin fixation. As far as examined in Lake Suwa on 4 March 1986, there was no large

Table 1. Comparison of three fixation procedures.

Immersion period	Air dried	Oven dried ^a	Formalin fixed ^b
5 min	0.74 ± 0.19^c	1.0 ± 0.45	0.73 ± 0.19
4 hrs	1.0 ± 0.30	0.98 ± 0.23	1.4 ± 0.30
7	2.0 ± 0.60	1.8 ± 0.23	2.0 ± 0.30
24	3.2 ± 1.4	3.6 ± 1.0	3.2 ± 0.74

^a ; At 40 C.

^b ; Glass slide was immersed in 2% formalin for a few minutes, rinsed with PFDW twice, and then air dried.

^c ; $\times 10^6$ cells $\cdot$ cm⁻².

difference in the attached bacterial number among the three fixations (Table 1). It was confirmed that the employed fixation technique was not the source of error in this study. The burning fixation technique, which was usually employed for the fixation of bacteria smeared on the glass slide, was not applied in this study in order to diminish the possible risk of collapse of the attached algal cells by the strong fixation.

We first applied this method in Lake Suwa, a shallow eutrophic lake in June 1985, when the number of planktonic bacteria determined by acridine orange direct count using a Nuclepore filter of 0.2 μ m pore size (HOBIE *et al.*, 1977) was

about 2×10^7 cells $\cdot$ ml⁻¹ (mean $\pm$ standard deviation were $2.13 \pm 1.36 \times 10^7$ cells $\cdot$ ml⁻¹ on 10 June and $1.83 \pm 0.84 \times 10^7$ cells $\cdot$ ml⁻¹ on 25 June). Figure 1 shows that the attached bacterial number amounted to over 10^5 cells $\cdot$ cm⁻² within several hours of immersion with the rate of $0.5 - 2 \times 10^5$ cells $\cdot$ cm⁻² $\cdot$ hr⁻¹. As shown in Table 1, however, a case in March evidenced a significant amount of attached bacteria on a glass slide of only 5 minute immersion, whereas hardly any were observed on the glass slide before immersion in the lake. Between 24 and 48 hr of immersion the attached bacterial number reached 10^6 cells $\cdot$ cm⁻² in June in Lake Suwa.

Fig. 1 Increase in number of attached bacteria (○) and the contribution of large cells to total attached bacteria in percent (●). A: Glass slides were immersed on 10 June; B: 12 June; C: 25 June. Bars indicate standard deviation in each count.

Fig. 2 Epifluorescence micrographs of immersed glass slides on 25 June ; (a) : a spread pattern of bacteria on the glass slide of 4-hour immersion ; (b) : a bacterial pattern observed at 48 hours ; (c) : bacteria covered with amorphous fluorescence observed at 24-hour immersion ; (d) : large bacterial aggregates found on the slide of 4-hour immersion.

Table 2. Total number of bacteria attached to immersed glass slide and frequency of dividing cells (FDC). Experiment was started on 25 June.

Immersion period (hrs)	Total bacteria (T, $\times 10^5 \text{ cells} \cdot \text{cm}^{-2}$)	Dividing cells (D, $\times 10^4 \text{ cells} \cdot \text{cm}^{-2}$)	FDC (D/T, %)
2	3.66	n. d.	n. d.
4	4.86	1.13	2.3
8	7.71	5.10	6.6
24	9.13	18.1	19.9

n. d. ; not determined.

Although it is a tentative definition of more than about 0.5 to 1.0 μm in length, the contribution of these large bacteria to the total population tended to increase with the immersion period (Fig. 2). The increase in cell size probably provided by the attachment to the solid surface will lead to higher attached bacterial per-cell activity for nutrient scavenging than that of free-living bacteria (HODSON *et al.*, 1981 ; PAERL and MERKEL, 1982).

The FDC has been proposed as an indicator of bacterial growth rate (HAGSTRÖM *et al.*, 1979 ; NEWELL and CHRISTIAN, 1981). Table 2 shows that the FDC in a bacterial population adsorbed on the glass slide increased significantly at 24 hr of *in situ* incubation, while the FDC observed at 4 or 8 hr are the number usually counted in this lake (OH, unpublished). This might suggest that bacteria grow at a higher rate on the surface of a glass slide than in bulk waters. The increase in FDC seemed to follow the increase in bacterial cell size as mentioned above.

Microscopic image of attached bacteria on the glass slide observed in June is given in Figure 2. Figure 2(a), (b) show two types of attached bacteria on glass surface : Figure 2(a) indicates a spread pattern observed for the sample of four hours of immersion. If this is produced by an activity of one or some bacterium/a, they would be a gliding type (NAGATA and WATANABE, 1983). Figure 2(b) is an aggregate, called "Chinese letter" by HIRSH (1984), which we observed at 48 hr. After 24 hr of immersion, usually it became difficult in Lake Suwa to count bacteria due to the strong fluorescence from attached algae and fluorescent crystals as shown in Figure 2(c).

Figure 2(d) is a photograph of large bacterial aggregates from the glass slide immersed in lakewater for four hours. Each aggregate contained a strongly fluorescent core substance of 20

or 30 μm in diameter. If these large aggregates were formed from a bacterium within four hours of immersion, the doubling time for these bacteria would be about 20 min, probably a Guinness Record for natural bacteria. Otherwise, the aggregates would have already been formed in water and floated as colonies. Although it was suggested that the growth rate of bacteria on the surface of glass slide might be larger than that in bulk water, the estimated doubling time of 20 min seems to be too short compared with the shortest doubling time of a few hours for planktonic bacteria observed in this lake (KATO, unpublished). Thus the latter explanation is more plausible in this case.

Bacterial attachment to the solid surface of a glass slide and their behaviour on it was easily and directly observed with epifluorescence microscopy by the method described here.

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摘 要

落射蛍光顕微鏡によるバクテリアの スライドグラスへの付着現象の観察

湖水中のバクテリアの固体表面 (スライドグラス) への付着の様子をアクリジンオレンジによる蛍光染色法を応用して観察した。6月に諏訪湖で観測した結果では、バクテリアのスライドグラスへの付着の初期速度は、およそ $1 \times 10^5 \text{ cells} \cdot \text{cm}^{-2} \cdot \text{hr}^{-1}$ で、24時間後には 1 cm^2 あたり 10^6 のバクテリアが認められた。付着バクテリア群集の中に、大型の細胞や分裂中の細胞の占める割合が、時間と共に増大する傾向がうかがわれた。

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