Arch. Microbiol. 122, 271–274 (1979)

Archives of
Microbiology

A Microautoradiographic Study of the Activity of Attached and Free-Living Bacteria

Madilyn Fletcher

Department of Environmental Sciences, University of Warwick, Coventry CV4 7AL, U.K.

Abstract. Microautoradiography, combined with epifluorescent microscopy, was used to evaluate the uptake of tritiated amino acids by a marine *Pseudomonas* sp. A comparison was made between the activity of bacteria free-living in the medium and bacteria which were attached to glass, polyethylene or polystyrene substrata. The proportion of active bacteria was lower for free-living cells (53–82%) and those attached to polystyrene (53–76%) than for those attached to glass (77–99%) or polyethylene (73–96%). For bacteria attached to glass, assimilated labelled substrate was retained within the cell over 3 h, whereas with polyethylene, labelled material was released from the cells and adsorbed on the surrounding substratum. Hence the physiological activity of attached bacteria depended on the chemical composition of the substratum.

Key words: Marine pseudomonad – Activity of – Bacterial attachment – Microautoradiography – Attachment substrata.

Many bacteria are able to attach to solid surfaces, and in almost any natural environment a significant proportion of the bacteria is found on surfaces, e.g. detritus, sediment particles and a variety of man-made structures (Corpe, 1970; Marshall, 1976). There is laboratory evidence that the activity of bacteria may be actually increased through the addition of solid materials, e.g. glass (Hendricks, 1974; ZoBell, 1943), clays (Jannasch and Pritchard, 1972; Stotzky, 1966a, b) and ion exchange resins (Harwood and Pirt, 1972; Kuwajima et al., 1957). In such studies, increase in activity has been indicated by an increase in numbers of suspended cells (Harwood and Pirt, 1972; Kuwajima et al., 1957; ZoBell, 1943), in the total respiratory rate of the culture (Hendricks, 1974; Stotzky, 1966a, b; ZoBell, 1943) or the rate of nutrient uptake or product synthesis (Hendricks, 1974; Kunc and Stotzky, 1970). It is often assumed that this increase in activity is due to an increase in numbers of attached bacteria, and that attachment offers some nutritional advantage to the organisms (ZoBell, 1943). For example, nutrients may be more concentrated at the attachment surface through adsorption, or physico-chemical conditions of the surface microenvironment (e.g. pH, E_h) may promote activity. However, there has been no direct evidence that the activity of attached bacteria differs from that of free-living cells, primarily because of the difficulty in differentiating between the activities of the two types of population.

The purpose of this investigation was twofold: (i) to determine whether the heterotrophic activity of bacteria which are attached to surfaces differs from that of their free-living counterparts, and (ii) to determine whether the chemical nature of the substratum affects activity; this was done by using different materials as attachment substrata. The method used for measuring activities of both attached and free-living cells was microautoradiography combined with epifluorescent microscopy.

Materials and Methods

Organisms and Growth Conditions. A marine *Pseudomonas* sp (NCMB 2021), whose attachment properties have been previously described (Fletcher, 1976, 1977; Fletcher and Floodgate, 1973, 1976; Fletcher and Loeb, 1976), was grown in 100 ml of medium (0.1% w/v peptone, 0.07% w/v yeast extract powder in seawater, pH 7.6) for 48 h at 15° C with shaking. The bacteria, which were then in stationary phase, were collected by centrifugation and resuspended in sterile (filtered, 0.2 μm porosity, and autoclaved) seawater to a concentration of $\sim 7 \times 10^7$ to 5×10^8 bacteria per ml.

Preparation of Attached and Free-Living Bacterial Populations. Two series of experiments were carried out; in the first, glass coverslips (Chance) and polyethylene film (Sterilin) were used as attachment

0302-8933/79/0122/0271/$01.00

substrata, whereas in the second series, glass coverslips and pieces of polystyrene (Sterilin petri dishes) were used, all approximately 1 cm^2 in area. The glass was cleaned by soaking for 48 h in ethanol with 3% (v/v) HCl, and the plastics were washed with detergent; all substrata were rinsed exhaustively with sterile distilled water. They were then transferred to the bacterial suspensions and left for 1–2.5 h to allow bacterial attachment. By balancing the time allowed for attachment with the concentration of suspended cells, the number of bacteria which became attached was large enough for microscopical observation, but not so closely crowded to make microautoradiographic analysis difficult (cf. Fletcher, 1977).

After the substrata were removed from the bacterial suspension, they were rinsed in running sterile seawater and placed in a 20-ml bottle containing 5 ml sterile seawater. Each bottle then contained one glass coverslip and one piece of either polystyrene or polyethylene. When placed in clean seawater, some bacteria were desorbed from the substrata and released into the medium. These bacteria comprised the free-living bacteria for the subsequent experiment, thus insuring that both free-living and attached bacteria were derived from the same population.

Incubation with Radioactive Substrate. The substrate for the microautoradiographic analysis was a mixture of fifteen tritiated amino acids (Radiochemical Centre, Amersham), the composition being approximately the same as a typical algal hydrolysate. This was added at concentrations of 2 or 10 µCi per ml to the 5 ml seawater containing substrata. Two controls were also prepared: (i) one with no added labelled amino acids, so that any physical or chemical reduction of the photographic emulsion silver halide which was not due to labelled substrate could be detected, and (ii) one to which tritiated amino acids were added but which was immediately fixed with 0.1 ml formalin, so that any adsorption of label by the cells could be detected and not confused with actual heterotrophic uptake.

The bottles were then incubated at 15° C with shaking, and at intervals of up to 3 h, the bacteria were fixed with 0.1 ml formalin and prepared for microautoradiography. Free-living cells were collected by filtering the suspension with twice its volume of sterile seawater through a polycarbonate filter (0.2 µm porosity) which previously had been stained with Irgalan black (Union Color and Chemical, Boston, Mass.) to prevent autofluorescence. The filters were mounted on slides by dipping the slides in a solution of 0.5% gelatin in distilled water (at approximately 45° C) and immediately placing the filter on the wet slide. The glass and plastic substrata were fixed on slides with double-sided adhesive tape.

The method used for the combined microautoradiography/epifluorescence was based on one developed by Dr. L.-A. Meyer-Reil, Institut für Meereskunde, Kiel (personal communication; Meyer-Reil, 1978). Mounted filters and substrata were coated with Kodak NTB2 nuclear track emulsion, diluted 1 : 3 in distilled water, and exposed for ~18 h at −30° C. For epifluorescent microscopy, staining with acridine orange was either (i) with a 0.02% w/v solution in 0.07 M phosphate buffer (pH 6.6) before development of the photographic emulsion, or (ii) with a 0.04% w/v solution in 0.2 M citrate buffer (pH 6.7) after development. Specimens stained with acridine orange in phosphate buffer were washed in phosphate buffer (×3), followed by distilled water. Those stained in citrate buffer were washed successively in citrate buffer of pH 6.7, 4.7, and 3.7, followed by distilled water. Both staining methods gave good results, but (ii) is preferable since fewer manipulations must be done in the dark. All specimens were developed at 20° C in Kodak D-19 developer (diluted 1:3 in distilled water), fixed with 30% w/v sodium thiosulfate in distilled water and thoroughly rinsed with tapwater. All manipulations of undeveloped specimens were done in total darkness or with a Kodak No 2 safelight filter.

After development and staining with acridine orange, specimens were air-dried and examined with blue incident-light excitation under a Zeiss Standard 18 microscope, using Cargille Type A immersion oil. The deposition of silver grains over, and in the immediate vicinity of a bacterium indicated that it had taken up tritiated amino acids. These labelled bacteria were counted and related to the total number of bacteria (determined by epifluorescence), giving the proportion of active cells. The number of silver grains associated with each labelled cell usually was too large to count, so that no attempt was made to evaluate rates of activity.

Results

Numbers of bacteria attached to the different substrata ranged between 0.2 and 6 per 100 μm^2. In experiments with glass and polystyrene, the number of bacteria attached to glass was between ~0.2 and 1.0 per 100 μm^2 and tended to remain the same over the 3 h of the experiment; the number of bacteria attached to polystyrene ranged between ~0.08 and 4.5 per 100 μm^2, and the lowest numbers reflected a reduction in the numbers of attached bacteria over the course of each experiment.

The proportion of labelled bacteria was lower for those free-living in the medium (53–82%) and those attached to polystyrene (53–76%) than for those attached to glass (77–99%) or polyethylene (73–96%) (Figs. 1, 2). After 1 h, almost all of the bacteria attached to glass showed uptake of labelled amino acids, and there was consistent heavy labelling, i.e. large numbers of silver grains were associated with these cells.

With bacteria attached to polyethylene, the labelled amino acids were taken up, but then much labelled material was quickly released,so that at 3 h incubation the silver grains were randomly distributed over the polyethylene surface and not particularly associated with the cells. This is shown in Fig. 3 by the counts of background silver grains, i.e. those which were not associated with cells; at 3 h incubation these are approximately three times higher for polyethylene substrata than for glass substrata or the filters containing the free-living bacteria. Examination of the fixed-cell controls confirmed that the high background counts on polyethylene were associated with bacterial activity and were not caused by physico-chemical adsorption of labelled amino acids. In separate experiments with polystyrene, background counts were similar to those obtained with glass.

Cells which did not take up labelled amino acids were also often morphologically distinct. Their nucleic acids were frequently condensed, usually towards the poles of the cells. Inactive cells were also often enlarged and intensely stained with acridine orange.

Discussion

The results of this investigation demonstrate that the proportion of bacteria which are heterotrophically

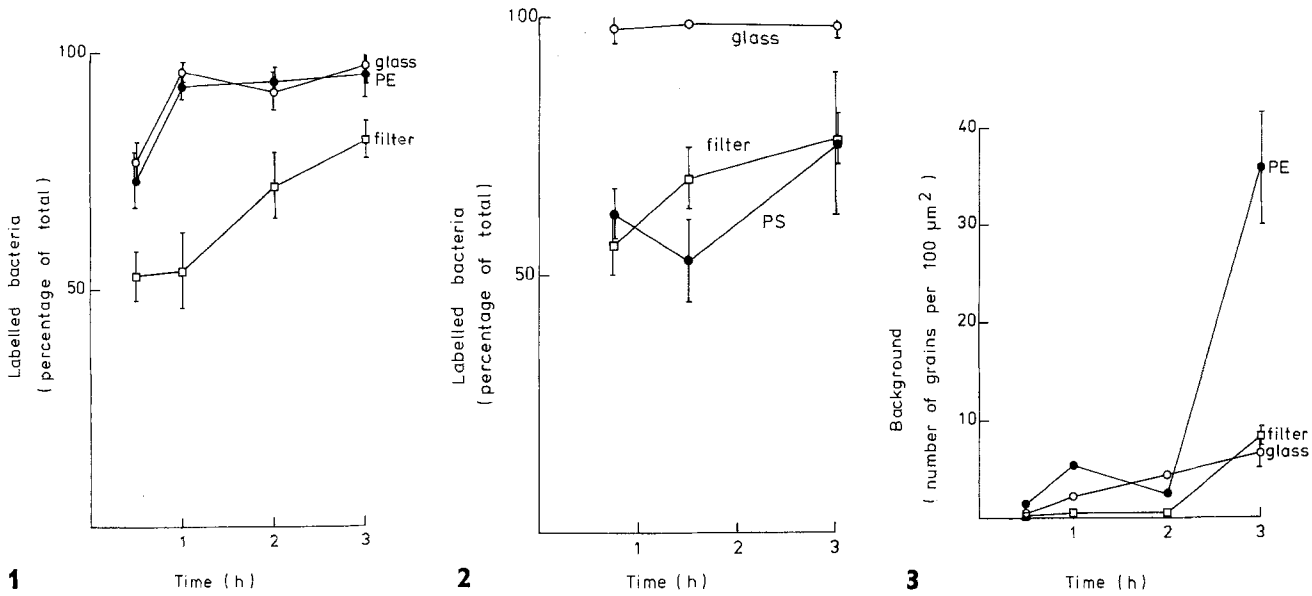

Fig. 1. The relationship between the proportion of bacteria assimilating labelled amino acids and time. After 1 h incubation, almost all of the bacteria attached to glass and to polyethylene (*PE*) have taken up the label, and the proportion of labelled cells is similar for both substrata. With the free-living bacteria (filter), however, the proportion of labelled cells was appreciably lower than that obtained with the attached bacteria

Fig. 2. The relationship between the proportion of bacteria assimilating labelled amino acids and time. As in Fig. 1, almost all of the bacteria attached to glass have taken up the label after 45 min. However, the proportion of labelled bacteria is appreciably less for bacteria attached to polystyrene (*PS*) and for the free-living bacteria (filter)

Fig. 3. The relationship between the number of background silver grains on glass and polyethylene (*PE*) substrata, on the filter containing free-living bacteria and time. Background grains were those which were not associated with cells. After 3 h, there is a considerable increase in background grains on the polyethylene (*PE*) substrata, as the bacteria released labelled material which was then adsorbed on the attachment surface

active on an attachment substratum can, but does not necessarily, differ from the proportion of active free-living bacteria (Figs. 1, 2). A higher proportion of active cells in a given microenvironment could suggest that the physical and chemical conditions of the environment are more favourable for active uptake of nutrients (Stotzky and Rem, 1966; ZoBell, 1943), but micro-autoradiography showed no adsorption (hence concentration) of amino acids on the substrata. The presence of inhibitory or stimulatory substances on a substratum could also affect activity of attached bacteria. This is particularly true for polyethylene and polystyrene, since there may be residual traces of chemicals used in their manufacture.

An alternative explanation for differences in activity on different substrata is that the conditions in environments with higher proportions of active cells may favour the removal of inactive cells; possibly inactive bacteria are more easily sloughed off a glass or polyethylene surface than off a polystyrene substratum. Since the tendency for this bacterium to become attached is dependent upon the composition of the substratum (Fletcher, 1976; Fletcher and Loeb, 1976), the strength of this attachment should also be affected by substratum characteristics, as well as the physiological condition and surface properties of the bacterium.

One of the most important points raised by this study is that the physico-chemical properties of different substrata may affect the activity of attached bacteria. Substratum composition affected not only the uptake of labelled amino acids (Figs. 1, 2) but also the length of time the label was retained within the cells (Fig. 3). The labelled material released by the bacteria attached to polyethylene could have been metabolic waste products or extracellular polymers. However at this stage it is impossible to explain why this material should be exuded, and not retained within the cells, as occurred on the glass substratum.

It is not surprising that the different microenvironments created on glass and on the polymer surfaces might promote differences in bacterial activity. Glass is a hydrophilic and highly active surface, able to enter into a number of types of interactions, e.g. dipole-dipole, electrostatic. However, the differences in activity on the two polymers are more difficult to interpret, since they are both hydrophobic with low surface activities (Andrade, 1973; Zisman, 1964). It has already been shown that this bacterium readily attaches

to both polyethylene and polystyrene, whereas it attaches to glass, and similar surfaces such as mica, in much lower numbers (Fletcher and Loeb, 1976). Thus there is no obvious correlation between the tendency for the bacterium to attach to a surface and its activity subsequent to attachment.

Therefore, although the results demonstrate that the heterotrophic activity of attached bacteria may be influenced by the substratum, it is not possible at this stage to predict from the chemical properties of the surface how it exerts this influence. It is likely that a wide range of factors can affect the system, including the adsorption of dissolved macromolecular substances and electrolytes on the substratum (Loeb and Neihof, 1977). The physiological state of the organisms should also be important. Stationary phase cells were used here, and different results could be expected with log phase or continuous culture organisms. Further studies investigating the influence of physiological condition, substratum composition and environmental factors on the activity of attached bacteria are in progress.

Acknowledgement. I am very grateful to Dr L.-A. Meyer-Reil for supplying the technique for combined epifluorescence and microautoradiography. Part of this work was carried out using a Zeiss Standard 18 microscope purchased with a Royal Society Scientific Investigations Grant.

References

Andrade, J. D.: Interfacial phenomena and biomaterials. Med. Instr. **7**, 110–119 (1973)

Corpe, W. A.: Attachment of marine bacteria to solid surfaces. In: Adhesion in biological systems (R. S. Manly, ed.), pp. 73–87. New York: Academic Press 1970

Fletcher, M.: The effects of proteins on bacterial attachment to polystyrene. J. Gen. Microbiol. **94**, 400–404 (1976)

Fletcher, M.: The effects of culture concentration and age, time and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. **23**, 1–6 (1977)

Fletcher, M., Floodgate, G. D.: An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. J. Gen. Microbiol. **74**, 325–334 (1973)

Fletcher, M., Floodgate, G. D.: The adhesion of bacteria to solid surfaces. In: Microbial ultrastructure (R. Fuller, D. W. Lovelock, eds.), pp. 101–107. London: Academic Press 1976

Fletcher, M., Loeb, G. I.: The influence of substratum surface properties on the attachment of a marine bacterium. In: Colloid and interface science, Vol. III (M. Kerker, ed.), pp. 459–469. New York: Academic Press 1976

Harwood, J. H., Pirt, S. J.: Quantitative aspects of growth of the methane oxidizing bacterium *Methylococcus capsulatus* on methane in shake flask and continuous chemostat culture. J. Appl. Bacteriol. **35**, 597–607 (1972)

Hendricks, C. W.: Sorption of heterotrophic and enteric bacteria to glass surfaces in the continuous culture of river water. Appl. Microbiol. **28**, 572–578 (1974)

Jannasch, H. W., Pritchard, P. H.: The role of inert particulate matter in the activity of aquatic microorganisms. Mem. Ist. Ital. Idrobiol. **29** Suppl., 289–308 (1972)

Kunc, F., Stotzky, G.: Breakdown of some aldehydes in soils with different amounts of montmorillonite and kaolinite. Folia Microbiol. **15**, 216 (1970)

Kuwajima, Y., Matsui, T., Kishigami, M.: The growth-supporting effect of some anion exchange resins for Phase I *Haemophilus pertussis*. Japan. J. Microbiol. **I**, 375–381 (1957)

Loeb, G. I., Neihof, R. A.: Adsorption of an organic film at the platinum-seawater interface. J. Mar. Res. **35**, 283–291 (1977)

Marshall, K. C.: Interfaces in microbial ecology. Cambridge, Mass.-London: Harvard U.P. 1976

Meyer-Reil, L.-A.: Autoradiography and epifluorescence microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl. Environm. Microbiol. **36**, 506–512 (1978)

Stotzky, G.: Influence of clay minerals on microorganisms. II. Effect of various clay species, homoionic clays, and other particles on bacteria. Can. J. Microbiol. **12**, 831–848 (1966a)

Stotzky, G.: Influence of clay minerals on microorganisms. III. Effect of particle size, cation exchange capacity, and surface area on bacteria. Can. J. Microbiol. **12**, 1235–1246 (1966b)

Stotzky, G., Rem, L. T.: Influence of clay minerals on microorganisms. I. Montmorillonite and kaolinite on bacteria. Can. J. Microbiol. **12**, 547–563 (1966)

Zisman, W. A.: Relation of equilibrium contact angle to liquid and solid constitution. Adv. Chem. Ser. **43**, 1–51 (1964)

ZoBell, C. E.: The effect of solid surfaces upon bacterial activity. J. Bacteriol. **46**, 39–56 (1943)

Received October 26, 1978