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Model System for Studies of Microbial Dynamics at Exuding Surfaces Such as the Rhizosphere

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An autoclavable all-glass system for studying microbial dynamics at permeable surfaces is described. Standard hydrophobic or hydrophilic membranes (46-mm diameter) of various pore sizes were supported on a glass frit through which nutrient solutions were pumped by a peristaltic pump. The pump provided a precisely controlled flow at speeds of 0.5 to 500 ml of defined or natural cell exudates per h, which passed through the membrane into a receiving vessel. The construction allowed a choice of membranes, which could be modified. The system was tested with a bacterium, isolated from rape plant roots (*Brassica napus* L.), that was inoculated on a hydrophilic membrane filter and allowed to develop into a biofilm. A defined medium with a composition resembling that of natural rape root exudate was pumped through the membrane at 0.5 ml/h. Scanning electron microscopic examinations indicated that the inoculum formed microcolonies embedded in exopolymers evenly distributed over the membrane surface. The lipid composition and content of poly-beta-hydroxybutyrate in free-living and adhered cells were determined by gas chromatography. The bacterial consumption of amino acids in the exudate was also studied.

Interactions between microorganisms and surfaces are complex and involve processes such as chemotaxis, adhesion, and production of extracellular polymers, in addition to community development leading to complex microbial surface films. Numerous model systems have been developed to study microorganisms on nonpermeable inert surfaces or living tissue surfaces (2, 5, 7, 8, 10, 16, 23). Martens (18) designed an artificial membrane system for the study of microbe-surface interactions in the rhizosphere.

Unlike inert, nonbiological surfaces, cell surfaces may exude metabolites and degradation products of both stimulatory and inhibitory qualities (9, 11, 25, 26, 30). These exudates specifically or nonspecifically influence microbial growth and community structure at the surface. Microbial colonizers may in their turn alter both the surface structure and the exudation characteristics (12, 14, 15, 19, 22).

Biochemical methods for assessing microbial biomass and community structure of biofilms are gaining interest as alternatives to traditional counting techniques (32). They have proved to be free of both the distortions induced with requirements for quantitative removal of organisms from surfaces and the selectivity introduced when organisms are required to grow on artificial media (31). For example, the total cellular biomass can be estimated from the content of phospholipids, and the fatty acid composition of the phospholipids fraction can be used to define the community composition (32). Microbial metabolic activity may be estimated by studies of the formation of the endogenous lipid storage product poly-beta-hydroxybutyrate (PHB) (6, 31).

We present an exuding surface model system developed to meet the following criteria. (i) The exuding surface should be permeable to relevant molecules, exhibit a well-defined pore size and hence produce uniform and reproducible flows and exudation characteristics, and must prevent microorganisms from entering the exudate reservoir. (ii) The surface should be inert towards living organisms. (iii) The area of the exuding surface and the total volume of the system should allow studies of initial attachment processes at natural substrate concentration and flows. (iv) Chemical signatures for microbial biomass and community structure (31) should be easily extractable from the various system environments with aqueous or organic solvents. (v) The system should permit increased levels of complexity in both biological and physical parameters, such as the use of soil instead of a liquid surrounding medium. (vi) The delivery system should allow controlled variations in the levels of exudate flow through the semipermeable surface. (vii) The system should be easily autoclavable. The model system was tested by using a bacterium isolated from the rhizosphere of the rape plant *Brassica napus* (L.).

MATERIALS AND METHODS

Model system. The system consisted of (i) a device for supplying exudate, including a membrane filter holder, (ii) a vessel with surrounding medium, and (iii) a flow-driving unit (Fig. 1).

The exudate solution was passed through a membrane fitted in a funnel-shaped glass holder with ground edges (A in Fig. 1). The diameter of the membrane holder was 50 mm, which allowed standard membrane filters (46 mm; Millipore Corp., Bedford, Mass.) or other filter brands to be used (B). Glass beads (3 mm) were sintered to the inside surface of the holder (A), providing support for the membrane and decreasing the holdup volume. An inlet capillary tube (130 by 6 mm; inner diameter, 2 mm), which fit snugly into a screw thread joint (Quickfit, Staffordshire, England; size 13) was attached to the holder. A glass ring (C) held the membrane filter to the edge of the holder. The all-glass assembly was secured with two strong stainless steel clips (D). The holdup volume was approximately 0.9 ml.

The vessel enclosing the liquid medium was constructed from a 600-ml beaker equipped with a flat flange lid (E). Several screw thread joints (F) were mounted in the lid and in the beaker wall. They allowed fittings to pass through, e.g., for the filter holder and thermometer, and provided air

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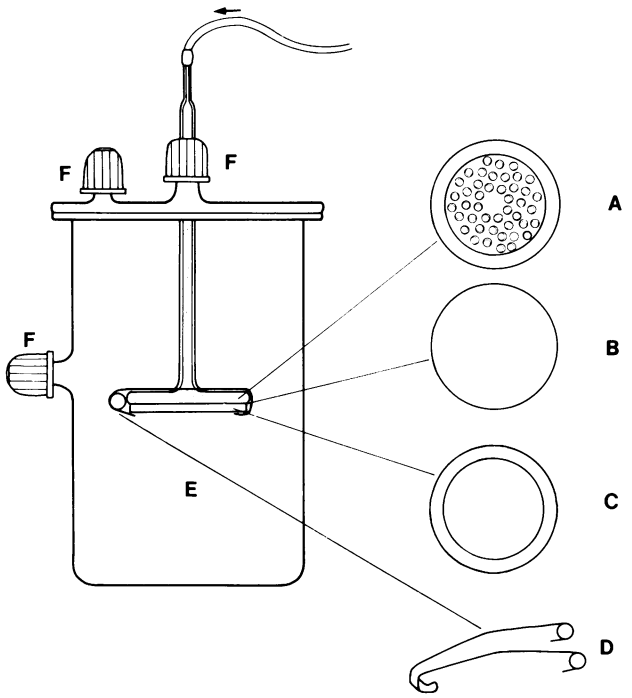

FIG. 1. Schematic drawing of membrane filter holder and sampling vessel.

ventilation and support for rubber septa to be penetrated for sampling.

The model system was driven with a dual channel peristaltic pump (LKB, Bromma, Sweden; model Microperpex), allowing exudate flow speeds of 0.5 to 500 ml/h. One LKB pump could thus drive two independent systems. A silicone tubing (inner diameter, 3 mm) connected the model system, pump, and exudate reservoir.

The complete system, including the medium, could be autoclaved after assembly. The membrane filter holder needed to be placed above the reservoir surface to avoid pressure fluctuations during autoclaving. Heat-labile components, for example, carbohydrate constituents, were filter sterilized and added after autoclaving. After sterilization, microbes were either added to the surrounding medium or associated with the membrane surface.

To obtain a constant flow of exudate, the tubing and the membrane filter holder needed to be completely filled with exudate, with no air bubbles. This was achieved after assembly in the following way. The medium from the receiving vessel was pumped through the membrane until it passed a T joint between the filter holder and the pump. During this operation, the third connection of the T joint was closed. Then the connection facing the filter holder was closed and the third connection was opened. Next, the pump was allowed to fill the tubing from the exudate reservoir. Then the third connection of the T joint was closed and the connection facing the filter holder was opened, producing a bubble-free exudate flow.

Organisms associated with the membrane were extracted by suspending the filters in suitable solvents, for example, for lipids, in chloroform-methanol. For small numbers of microorganisms, the filters were preextracted with the solvents, dried, and thoroughly washed with sterile water or buffer before the experiment. The reservoir medium was sampled with a syringe by penetrating a gas chromatography (GC) septum (12-mm diameter) housed in a screw thread joint in the vessel wall.

Biofilm experiment. The model system was tested by using a strain of a gram-negative bacterium isolated from the roots of the rape plant *Brassica napus* (L.) (28). The bacteria were cultured on a defined medium containing 2.0 g of glucose, 2.0 g of sucrose, and 0.2 g of an amino acid mixture in 0.1 M HCl per liter of buffer. The buffer solution consisted of 0.7 g of K_2HPO_4, 0.3 g of KH_2PO_4 (pH 6.5), and 0.01 g of $MgSO_4$ per liter. The composition of the amino acid mixture was 28.6% (wt/vol) glycine, 17.0% alanine, 4.2% valine, 1.9% leucine, 2.6% isoleucine, 1.0% phenylalanine, 0.9% cysteine, 16.7% aspartatic acid, 13.2% glutamic acid, 4.0% proline, 8.9% lysine, and 1.0% arginine. The composition of this medium was based on data from analyses of carbohydrates and amino acids in exudates from sterile rape roots (10; A. Tunlid, unpublished data). The bacteria were incubated with shaking at room temperature for 48 h. At the end of the incubation period, the density was 3×10^8 cells per ml.

The filters used were hydrophilic Durapore membrane filters (Millipore; polyvinylidene fluoride; pore size, 0.22 μm; diameter, 46 mm). After sterilization, the membrane filter holder was placed in 100 ml of the bacterial suspension, and the bacteria were allowed to attach to the membrane surface for 2 h at room temperature, during which time the suspension was slowly agitated. Then the membrane was rinsed twice with the buffer solution to remove loosely attached organisms.

The membrane filter holder with attached bacteria was fitted to the lid of the vessel (A in Fig. 1). The surrounding medium consisted of buffer. The exudate consisted of sucrose, glucose, and the amino acid mixture at concentrations in the buffer 0.4 times those of the previously described defined medium. The flow of exudation was adjusted to 0.5 ml/h. The uptake and metabolism of the amino acids in the exudate by the initially attached population were studied during the subsequent 10 h. Samples (5 ml) of the surrounding medium were taken initially (0 h) and after 10 h of exudation.

After sampling, exudation was allowed to proceed for 7 days. The free-living bacteria were harvested by centrifugation (15 min at 10,000 × *g*). The membrane filter holder was rinsed with sterile buffer and placed in another sterile vessel with 350 ml of buffer. Samples of amino acids were taken at 0 and 10 h.

In a control experiment, a membrane without attached bacteria was used. Samples of the medium were taken for amino acid analysis at 0 h and after 10 h of exudation.

Analysis. (i) Amino acid analysis. Norleucine (948 ng) was added to the samples as an internal standard. The amino acids were converted to the *N*-heptafluorobutyryl isobutyl esters and analyzed by capillary GC (1).

(ii) Lipid analysis. Freeze-dried membranes and bacterial pellets were extracted by the method of Bligh and Dyer (3) for phospholipids and PHB by the addition of 3 ml of a one-phase chloroform-methanol-water (2:1:0.8 [vol/vol/vol]) mixture. The extracts were supplemented with 2.0 μg of 3-OH 9:0 and 3.6 μg of 19:0 acids (internal standards). (Fatty acids in this study were designated as the number of total carbon atoms:number of unsaturation. Prefixes are as follows: *a*, ante-isobranching; *i*, isobranching; OH, hydroxyl with the position indicated.) The lipids and PHB were hydrolyzed, and the acids were worked up and purified as previously described (21). Acetonitrile (30 μl) was added, followed by 10 μl of 35% pentafluorobenzyl bromide in acetonitrile and 10 μl of triethylamine (modified from that

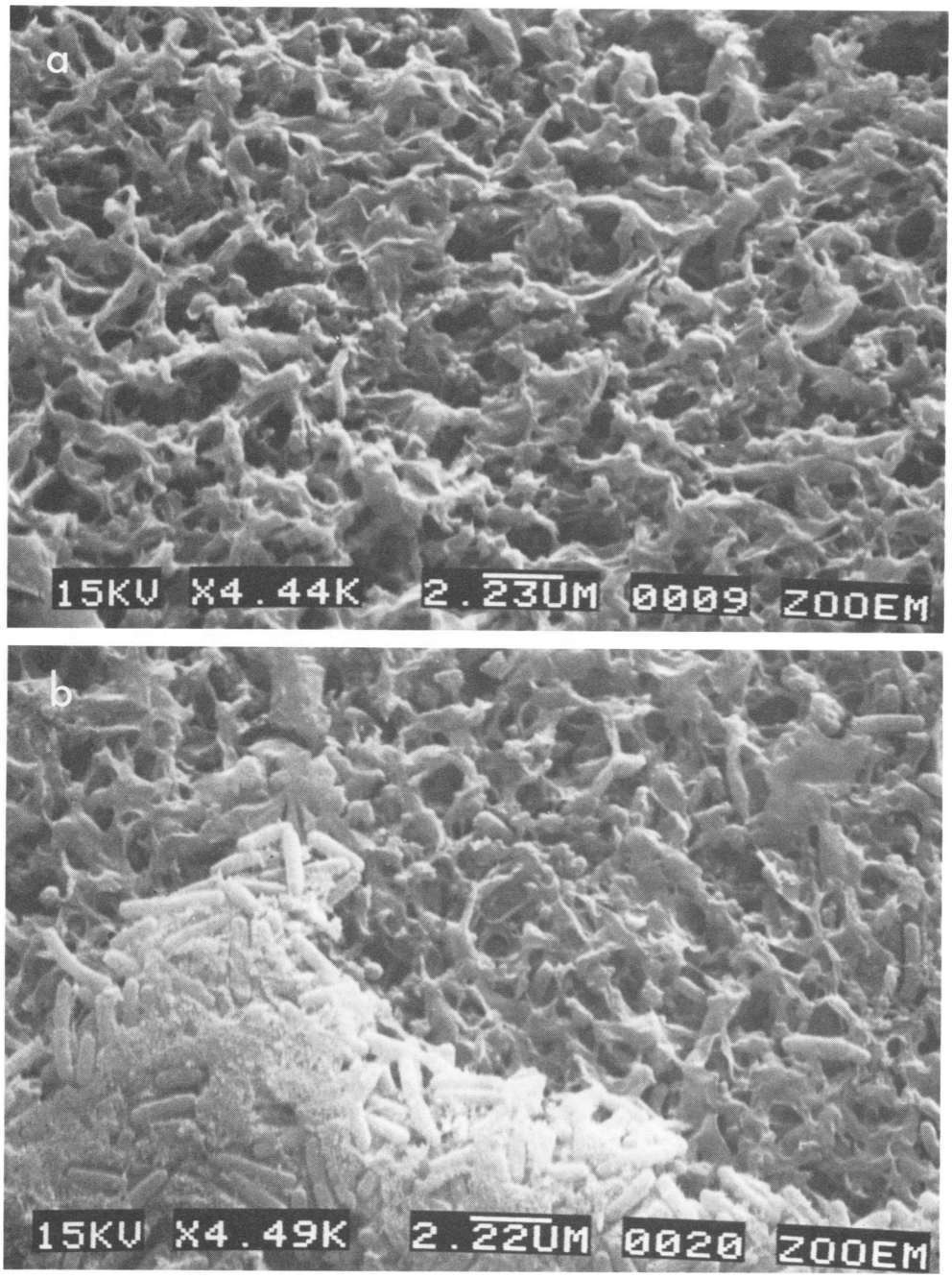

FIG. 2. Scanning electron micrographs of initially attached bacteria (a) and biofilm developed after 7 days (b). Bars, 2 μm.

described in reference 27). After 10 min at room temperature, 300 μl of hexane was added, and a 1-μl portion was analyzed by GC.

(iii) GC. The fatty acid and amino acid derivatives were analyzed on a 25-m fused silica capillary column (inner diameter, 0.2 mm), coated with methylphenyl vinyl silicone (SE-54) as stationary phase, by using a Varian model 3700 instrument equipped with a flame ionization detector and in conjunction with a Hewlett-Packard 3390A reporting integrator. H_2 served as the carrier gas, at a flow rate of 60 cm/s, and N_2 was used as the makeup gas, at a flow rate of 30 ml/min. Injections were performed in a splitless mode.

The gas chromatographic parameters were as follows. Fatty acids: injection temperature, 250°C; detector temperature, 275°C; temperature programming, 90°C for 2 min, then 4°C/min to 275°C; the split valve was opened after 60 s. Amino acids: injection temperature, 250°C; detector temperature, 275°C; temperature programming, 80°C to 250°C at a rate of 5°C/min; the split valve was opened after 30 s.

(iv) Scanning electron microscopy. Durapore filter membranes (Millipore) with attached bacteria were fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) for 2 h at room temperature. The filters were dehydrated in acidified dimethoxypropane, washed in absolute alcohol, and transferred to Freon TF (E. I. du Pont de Nemours & Co., Inc.). The air-dried specimens were mounted on holders and

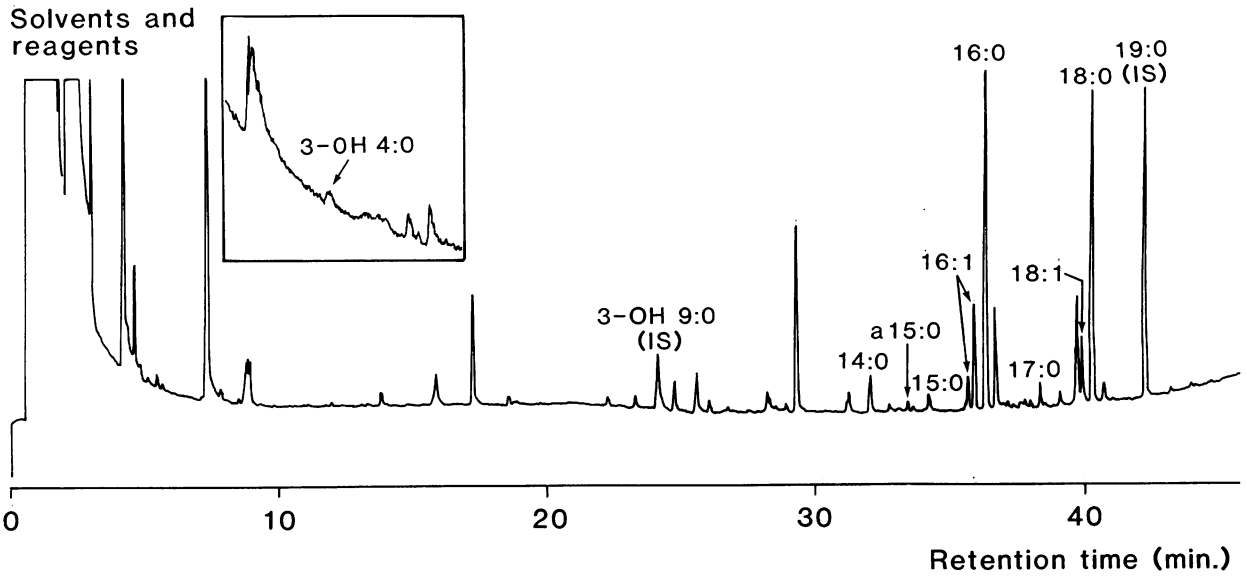

FIG. 3. Capillary GC of PFB-esters of fatty acids derived from phospholipids of initially attached rape root bacteria. Inset shows part of a GC (PFB-derivative of 3-hydroxybutyric acid) at four times higher sensitivity. IS, Internal standards.

sputtered with gold-palladium. The preparations were examined in a Nanolab 2000 instrument.

(v) **Solvents, standards, and reagents.** Solvents were of reagent grade and were not redistilled before use. The 2,3,4,5,6-pentafluorobenzylbromide and heptafluorobutyric anhydride (all purities >99%) were from Fluka AG, Buchs, Switzerland.

The fatty acid standards, 3*R*-hydroxynonanoic acid (3-OH 9:0), 3*S*-hydroxydodecanoic acid (3-OH 12:0), and nonadecanoic acid (19:0), were from the Lund laboratory collection (purity, >99%). Racemic 3-Hydroxybutyric acid (purity, >97%) was from Fluka AG.

RESULTS AND DISCUSSION

Model system. During initial experiments, the C_x-10-ultrafilter (Pellicon; Millipore), used by Martens (18) as an "artificial root," was tested. Although the membrane filter showed no selectivity in retention when testing a mixture consisting of D-(+)-glycose, carbon dioxide, and L-alanine, it was very difficult to clean and sterilize. Furthermore, neither the filter nor the support structure for the filter was compatible with the organic solvents used for extraction of the lipids in the biofilm.

This led us to develop the apparatus illustrated in Fig. 1. The device permitted the use of a wide range of membranes with different surface characteristics. For example, membranes with different pore sizes (ultrafilters and filters for reversed osmosis) could be used to cut off high-molecular-weight components in natural exudates. The surface material could be chosen or modified to resemble natural tissue surfaces by coating with phospholipids and polysaccharides. Possibly, certain genuine tissues or films of cultured cells could be placed in the holder.

More advanced delivery systems could allow gradients or pulsed administrations of exudates. Exudate components labeled with stable isotopes such as ^{13}C or ^{15}N would provide opportunities to study substrate uptake and metabolism at the surface by using mass spectrometric measurements of metabolites, without the disadvantages involved when radioactive isotopes are used (29).

Application. The rhizosphere bacteria adhered to the membrane surface and grew on the exudate from individual cells to a biofilm within 7 days (Fig. 2a and b). The scanning electron microscopy examination showed that the developed film resembled bacteria associated with plant roots in several characteristics (4, 24). The bacteria formed microcolonies embedded in extracellular polymers that covered less than 10% of the membrane surface.

The fatty acids ester-linked in the phospholipids were determined in the cells of the inoculum, in the initially attached cells, in the film formed after 7 days of growth, and in the unattached cells. The fatty acid profile of the initially attached bacteria was examined (Fig. 3). The analysis indicated shifts in the proportions of saturated and unsaturated fatty acids in cells from different environments. For example, the ratio of 18:1 to 18:0 was higher in the free-living cells than in cells associated with the surface. Dramatic and rapid shifts in the ratio between unsaturated and saturated fatty acids due to environmental changes have recently been reported by Malmcrona-Friberg et al. (17).

The rhizosphere bacteria used in the experiment accumulate PHB during growth in the defined medium (29). In our experiment, the initially attached bacteria contained PHB (80 ng/μg of total amount of fatty acids), but the level was lower relative to the total amount of fatty acids in cells of the biofilm (40 ng/μg of total amount of fatty acids). This decrease paralleled an experiment with rhizosphere bacteria grown with rape plant seedlings, in which microbes recovered with the roots showed increases in bacterial fatty acids but no formation of PHB (28). The unattached, free-living bacteria contained 0.01 ng of PHB per μg of total amount of fatty acids and probably did use PHB as an energy source, since the level of carbon and nutrients in the surrounding medium was very low due to the consumption of exudates by the surface-associated bacteria. It has been demonstrated that bacteria can utilize PHB during starvation conditions (17).

The biofilm bacteria and also to a certain extent the initially attached bacteria differentially consumed the amino acids in the exudate (Fig. 4A through C). Compared with the control experiment without bacteria, the initially attached

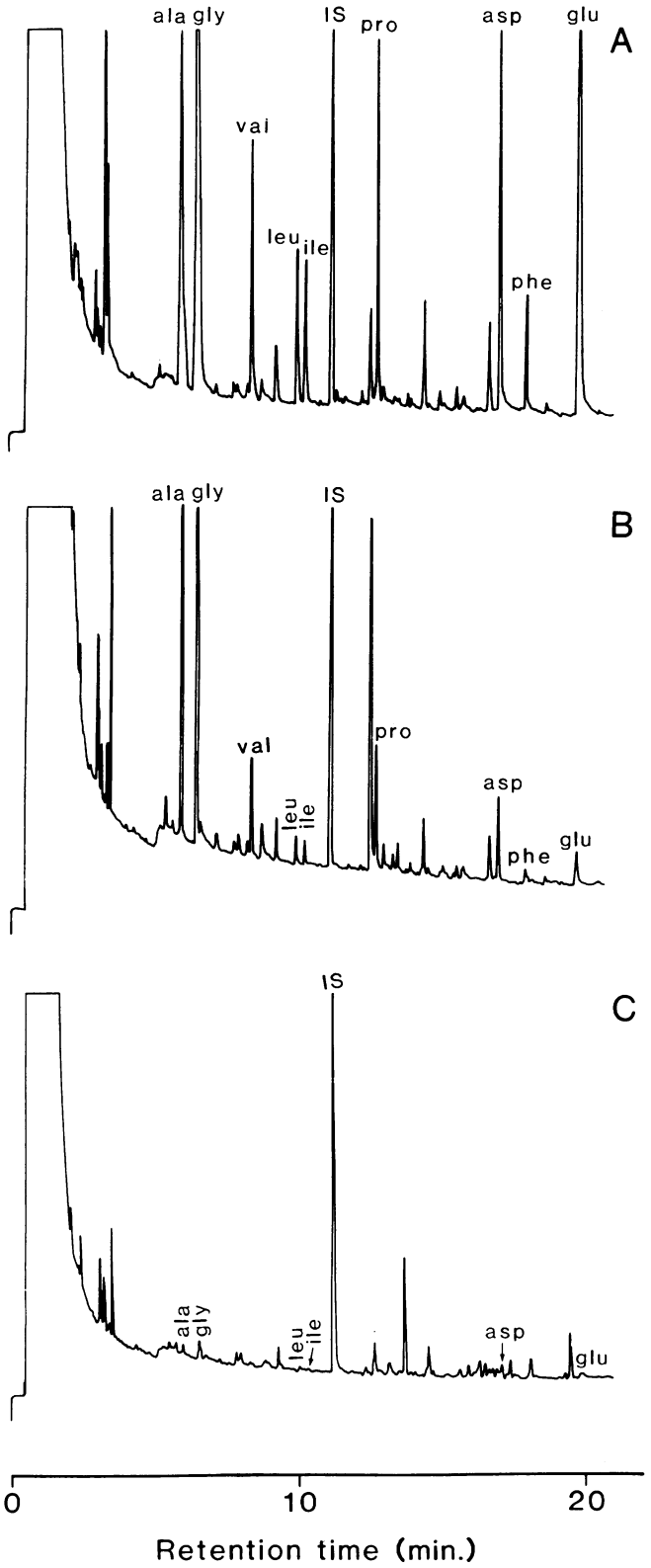

FIG. 4. Capillary gas chromatograms of *N*-heptafluorobutyryl isobutyl ester derivatives of amino acids in artificial rape root exudate. IS, Internal standard (norleucine). (A) Composition of original exudate in surrounding medium (control); (B) composition of exudate in surrounding medium modified by initially attached population; (C) composition of exudate in surrounding medium modified by biofilm.

population consumed about 85% of the amino acids in the original exudate. The bacteria in the 7-day biofilm used essentially all amino acids, with 1% of the total amount in the original exudate reaching the surrounding medium. This resulted in an amino acid concentration in the medium near the background level (below 10 ppb [10 ng/g]). The bacteria attached to the membrane changed the relative proportions of the amino acids in the original exudate. Aspartate and glutamate were more readily metabolized than alanine and glycine. The concentration of the exudate used in the experiment was within the range given for natural root exudates by Newman and Watson (20). Consequently, microorganisms situated outside the surface biofilm received a drastically modified exudate. This effect can be of great relevance for the interactions between pathogens and plant roots. For example, sugar and amino acid exudates are known to stimulate the germination of soil-borne fungal propagules (13).

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LITERATURE CITED

1. **Bengtsson, G., and G. Odham.** 1979. A micromethod for the analysis of free amino acids by gas chromatography and its application to biological systems. Anal. Biochem. **92:**426–443.
2. **Bennett, R. A., and J. M. Lynch.** 1981. Bacterial growth and development in the rhizosphere of gnotobiotic cereal plants. J. Gen. Microbiol. **125:**95–102.
3. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:** 911–917.
4. **Campbell, R., and A. D. Rovira.** 1973. The study of the rhizosphere by scanning electron microscopy. Soil Biol. Biochem. **5:**747–752.
5. **Candy, D. C. A., T. S. M. Leung, A. D. Phillips, J. T. Harries, and W. C. Marshall.** 1981. Models for studying the adhesion of enterobacteria to the mucosa of the human intestinal tract. Ciba Found. Symp. **80:**72–93.
6. **Dawes, E. A., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in microorganisms, p. 135–267. *In* A. H. Rose and D. W. Tempest (ed.), Advances in microbial physiology, no. 10. Academic Press, Inc. (London), Ltd., London.
7. **Duxbury, T., B. A. Humphrey, and K. C. Marshall.** 1980. Continuous observation of bacterial gliding motility in a dialysis microchamber: the effects of inhibitors. Arch. Microbiol. **124:** 169–175.
8. **Fletcher, M.** 1977. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. **23:**1–6.
9. **Gaworzewska, E. T., and M. J. Carlile.** 1982. Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. J. Gen. Microbiol. **128:**1179–1188.
10. **Hägquist, M.-L., H. Svenningsson, S. Olsson, P. Sundin, G. Odham, and C. Liljenberg.** 1984. Long-term culturing of plants with aseptic roots. Determination of rape root exudates. Plant Cell Environ. **7:**549–552.
11. **Hale, M. G., L. D. Moore, and G. J. Griffin.** 1978. Root exudates and exudation, p. 163–203. *In* Y. R. Dommergues and S. V. Krupa (ed.), Interactions between non-pathogenic soil microorganisms and plants. Elsevier Scientific Publishing Co., Amsterdam.
12. **Hohmann, A., and M. R. Wilson.** 1975. Adherence of enteropathogenic *Escherichia coli* to intestinal epithelium in vivo. Infect. Immun. **12:**866–880.
13. **Krigsvold, D. T., G. J. Griffin, and M. G. Hale.** 1982.

Microsclerotial germination of *Cylindrocladium crotalariae* in the rhizospheres of susceptible and resistant peanut plants. Phytopathology **72**:859–864.

14. **Lange, S., H. A. Hansson, and I. Lönnroth.** 1983. Influence of bile acids on cholera toxin-induced secretion in mouse jejunum. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **91**:215–229.
15. **Lee, K. J., and M. H. Gaskins.** 1982. Increased root exudation of ^{14}C-compounds by sorghum seedlings inoculated with nitrogen-fixing bacteria. Plant Soil **69**:391–399.
16. **Mackowiak, P. A., and M. Marling-Cason.** 1984. A comparative analysis of in vitro assays of bacterial adherence. J. Microbiol. Methods **2**:147–158.
17. **Malmcrona-Friberg, K., A. Tunlid, P. Mårdén, S. Kjelleberg, and G. Odham.** 1986. Chemical changes in cell envelope and poly-β-hydroxybutyrate during short term starvation of a marine bacterial isolate. Arch. Microbiol. **144**:340–345.
18. **Martens, R.** 1982. Apparatus to study the quantitative relationships between root exudates and microbial populations in the rhizosphere. Soil Biol. Biochem. **14**:315–317.
19. **Martin, J. K.** 1977. Factors influencing the loss of organic carbon from wheat roots. Soil Biol. Biochem. **9**:1–7.
20. **Newman, E. I., and A. Watson.** 1977. Microbial abundance in the rhizosphere: a computer model. Plant Soil **48**:17–56.
21. **Odham, G., A. Tunlid, G. Westerdahl, L. Larsson, J. B. Guckert, and D. C. White.** 1985. Determination of microbial fatty acid profiles at femtomolar levels in human urine and the initial marine microfouling community by capillary gas chromatography - chemical ionization mass spectrometry with negative ion detection. J. Microbiol. Methods **3**:331–344.
22. **Ogawa, T., S. Kotani, M. Tsujimoto, S. Kusumoto, T. Shiba, S. Kawata, and K. Yokogawa.** 1982. Contractile effects of bacterial cell walls, their enzymatic digests, and muramyl dipeptides on ileal strips from guinea pigs. Infect. Immun. **35**:612–619.
23. **Olsson, J., and B. Krasse.** 1976. A method for studying adherence of oral streptococci to solid surfaces. Scand. J. Dent. Res. **84**:20–28.
24. **Rovira, A. D., and R. Campbell.** 1974. Scanning electron microscopy of microorganisms on the roots of wheat. Microb. Ecol. **1**:15–23.
25. **Rutter, J. M., and G. W. Jones.** 1973. Protection against enteric disease caused by *Escherichia coli* - a model for vaccination with a virulence determinant? Nature (London) **242**:531–532.
26. **Sakata, T., and W. van Engelhardt.** 1981. Luminal mucin in the large intestine of mice, rats and guinea pigs. Cell Tissue Res. **219**:629–635.
27. **Strife, R. J., and R. C. Murphy.** 1984. Preparation of pentafluorobenzyl esters of arachidonic acid lipoxygenase metabolites. Analysis by gas chromatography and negative ion chemical ionization mass spectrometry. J. Chromatogr. Biomed. Appl. **305**:3–12.
28. **Tunlid, A., B. H. Baird, S. Olsson, R. H. Findlay, G. Odham, and D. C. White.** 1985. Determination of phospholipid ester-linked fatty acids and poly-beta-hydroxyalkanoate for the estimation of bacterial biomass and activity in the rhizosphere of the rape plant *Brassica napus* (L.). Can. J. Microbiol. **31**:1113–1119.
29. **Tunlid, A., G. Odham, R. H. Findlay, and D. C. White.** 1985. Precision and sensitivity of the measurement of ^{15}N enrichment in D-alanine from bacterial cell walls using positive/negative ion mass spectrometry. J. Microb. Methods **3**:237–245.
30. **Tyagi, V. K., and S. K. Chauhan.** 1981. The effect of leaf exudates on the spore germination of phylloplane mycoflora of chilli (*Capsicum annuum* L.) cultivars. Plant Soil **65**:249–256.
31. **White, D. C.** 1983. Analysis of microorganisms in terms of quantity and activity in natural environments, p. 37–66. *In* J. H. Slater, R. Whittenburg, and J. W. T. Wimpenny (ed.), Microbes in their natural environments. Society for General Microbiology Symposium Series, no. 34. Cambridge University Press, Cambridge.
32. **White, D. C.** 1984. Chemical characterization of films, p.159–176. *In* K. C. Marshall (ed.), Microbial adhesion and aggregation. Dahlem Konferenzen 1984. Springer-Verlag KG, Berlin.