Microb Ecol (1983) 9:215–226

MICROBIAL ECOLOGY

Amino Acid Assimilation and Respiration by Attached and Free-living Populations of a Marine *Pseudomonas* sp.

John J. Bright* and Madilyn Fletcher

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

Abstract. The uptake kinetics of leucine and the assimilation and respiration of leucine, glycine, glutamate and arginine by a marine *Pseudomonas* sp. was evaluated to determine whether the uptake and efficiency of substrate utilization of free-living bacteria differed from that of bacteria associated with surfaces. Bacteria were allowed to attach to plastic substrata with known hydrophilicities, as measured by advancing water contact angle (θ_A); these were Thermanox, poly(vinylidene fluoride), poly(ethelene) and poly(tetrafluoroethylene). The assimilation and respiration of surface-associated bacteria depended on the amino acid and substratum θ_A, but assimilation by surface-associated cells was generally greater than and respiration was generally less than that by free-living bacteria. The uptake kinetics with leucine demonstrated that the half saturation constant (K) of surface-associated bacteria was greater than that for free-living cells. The V_{max} values for surface-associated and free-living bacteria were similar, except for cells associated with poly(tetrafluoroethylene), which had a higher V_{max} value.

Introduction

In many natural waters, especially those with high concentrations of particulate matter, a significant proportion of bacteria are found attached to solid surfaces [27, 32, 34]. Moreover, even if the population of attached organisms is small compared with free-living bacteria, they may be metabolically more active and thereby make an appreciable contribution to total microbial processes in a given aquatic system.

Some studies with pure cultures have indicated that inert surfaces can increase metabolic activity (e.g., substrate assimilation, respiration, growth) of attached microorganisms [4, 8, 23, 25], although others have shown that attachment may also decrease activity [6, 18, 19]. In the few studies where activity per cell of natural attached and free-living microbial populations have been directly compared, attached organisms were frequently found to be more active [2, 14, 16, 27]. Furthermore, in these and other more general evaluations of relative

* *Present address:* Dept. of Microbiology, The University, Leeds LS2 9JT, U.K. Send offprint requests to: M. Fletcher at the above address.

activities of natural attached and free-living populations [1, 17, 37], an underevaluation of the periphytic contribution was possible due to detachment of loosely held particle-associated organisms, during separation by size-fractionation or centrifugation [37]. As certain bacterial types may become selectively attached [5, 29, 40], it was also not always clear whether differences in activities of attached and free-living populations were due to differences in species composition.

There is evidence that the activity of surface-associated bacteria may be affected by conditions at the solid surface, and a number of these will differ from the bulk phase. For example, the microenvironment at the interface may be influenced by substratum surface charge, affecting the concentration of ions and molecules, and hence pH [19, 30], E_h [21], and substrate concentration [19]. Moreover, the uptake and release of molecules and/or ions by attached bacteria may be influenced by a modification of cell surface potential [39] and/or chemiosmotic processes [7]. Adsorption of large molecules, particularly enzymes, may produce beneficial [28, 35, 36] or disadvantageous [15, 36] conformational or steric effects. Adsorption or entrapment in bacterial exopolymers [14, 39] may also concentrate nutrients [29], exoenzymes, metabolites [39] or inhibitors [6, 18], and diffusion of these molecules towards, or away from, attached bacteria may be affected by the solid/liquid interfacial stationary layer [15].

Thus, the physicochemical properties of the attachment surface are probably important factors in determining the comparative activities of attached and free-living bacteria. The purpose of this study was to investigate the influence of surfaces on bacterial activity by (1) comparing the uptake kinetics and efficiency of substrate utilization of surface-associated and free-living bacteria and (2) evaluating the influence of substratum and substrate properties on these activities.

Methods

Organism and Preparation of Cell Suspensions

A marine *Pseudomonas* sp. was cultured in 100 ml of medium for 40 h at 15°C in an orbital incubator (150 rpm). The medium comprised 0.1% (w/v) peptone and 0.07% (w/v) yeast extract powder in artificial seawater (ASW) [26], pH 8.1. The cells, which were in stationary phase, were harvested by centrifugation and washed twice in ASW. The final washed cell suspension was adjusted to $\simeq 2 \times 10^8$ ml ASW^{-1}. For further details on the attachment properties of this organism, refer to references [9–13].

Substrata

The substrata were 15 or 16 mm diameter disks of the following: Thermanox (T) (Lip Services Ltd, Shipley, England), poly(vinylidene fluoride) (PVDF) (Solef, Solvay and Cie Société Anonyme, Brussels), poly(ethylene) (PE) (Storey Brothers, Manningtree, England), and poly(tetrafluoroethylene) (PTFE) (Dalau Specialised Plastics Ltd, Clacton-on-Sea, England). Refer to reference [4] for further details on substratum properties and evaluation of surface hydrophilicity by measurement of

advancing contact angles (θ_A) of water. The measured water θ_As for the surfaces used in this study were T, 59.4°; PVDF, 77.3°; PE, 93.6°; PTFE, 103.5°[4]. PVDF, PE, and PTFE disks were cut from sheets with a cork borer and cleaned by sonication for 1 h in filter sterilized distilled water.

Attachment of Bacteria to Substrata

Six replicate substrata of each material were prepared for each experiment, three each for activity measurements and cell counts. Surfaces were held by rings cut from silicone rubber tubing (14 mm internal diameter × 20 mm external diameter, ≃10 mm length) and placed upright in 25 ml universal screw-cap bottles. Seven milliter portions of washed cell suspension were placed in the bottles containing the amino acid for 2 h at 15°C to allow attachment. The substrata were then washed with 100 ml of filter sterilized ASW, at a flow rate of ≃200 ml min^{-1}, to remove residual suspended and loosely attached cells. The 6 replicate surfaces with attached cells were then secured in fresh sterile silicone rubber rings and transferred to 40 ml of incubation solution (see below) contained in a 100 ml conical flask and sealed with an air-tight rubber seal.

Incubation of Attached and Free-Living Bacteria with ^{14}C-Amino Acids

Attached and free-living bacteria were incubated for 2 h in an orbital shaker (75 rpm) at 15°C in the appropriate medium (see below). During incubation, some of the initially attached bacteria desorbed from the substrata, and these were collected by filtration (see below). These are referred to as the detached population, and attached and detached bacteria are referred to collectively as surface-associated cells. To prepare suspensions of free-living bacteria of similar numbers to the attached cell preparations, 3 replicate 1 ml portions of the washed cell suspension were placed in 40 ml of a corresponding incubation solution and incubated in the same way as the attached populations. Numbers of free-living bacteria before and after incubation were similar, indicating there was negligible attachment to the surfaces of the incubation flasks.

The media comprised ASW supplemented with NH_4Cl and KH_2PO_4 to produce a carbon-limited medium with a C:N:P ratio of 10:4:1. The carbon source was either (1) 10 μg carbon l^{-1} of L-[U-^{14}C] leucine (50 μCi ml^{-1}, 340 mCi $mmol^{-1}$), (2) 10 μg C l^{-1} L-[U-^{14}C] leucine with 90, 490, or 990 μg C l^{-1} unlabeled leucine carrier, giving total concentrations of 10, 100, 500, and 1,000 μg C l^{-1} or (3) 10 μg C l^{-1} [1-^{14}C] glycine (200 μCi ml^{-1}, 53.4 mCi $mmol^{-1}$), L-[U-^{14}C] glutamic acid (50 μCi ml^{-1}, 282 mCi $mmol^{-1}$) or L-[U-^{14}C] arginine monohydrochloride (50 μCi ml^{-1}, 345 mCi $mmol^{-1}$) (all supplied by Radiochemical Centre, Amersham, England). All incubation solutions were filter sterilized.

Evaluation of Respiration and Assimilation

At the end of the incubation period, the attached, detached, and free-living bacteria (3 replicates of each) were fixed by acidification of the incubation solution to pH 1.9 with 0.3 ml 1 M H_2SO_4 injected through the rubber seal which also released ^{14}C-CO_2. ^{14}C-CO_2 was absorbed by 2-phenylethylamine (Fisons, Loughborough, England) which had been applied by injection to a GF/G glass fiber wick in a well in the center of the flask immediately before acidification. A further 2 h incubation period at 15°C with shaking was allowed for ^{14}C-CO_2 absorption. Similar experiments, but without cells and substituting ^{14}C-NaH_2CO_4 for the ^{14}C-substrates, indicated that ≃100% of the ^{14}C-CO_2 was absorbed by the wicks after 2 h. Control surfaces without cells were incubated as above with ^{14}C-substrates to detect nonbiological ^{14}C-substrate adsorption onto the surfaces, ^{14}C-CO_2 release and sterility of the solutions. ^{14}C-amino acid adsorption on the substrata was negligible. Wicks with absorbed ^{14}C-CO_2 were placed into vials containing 10 ml Filter-solv™ (Beckman, High Wycombe, England).

To measure assimilation of amino acids, 3 replicate attached, detached, and free-living samples from each incubation solution were prepared. Surfaces with attached cells were drained but not washed, to avoid removal of attached cells. They were placed into vials containing 1 ml Lumasolve (LKB, Croydon, England) and 200 μl sterile distilled water and incubated for 12 h at 50°C to digest the cells before adding 10 ml Lipoluma scintillation liquid (LKB). To collect free-living and detached cells, six 5 ml portions from the incubation solutions containing free-living cells or which had contained the substrata were filtered through polycarbonate filters (0.2 μm porosity; Bio-Rad, Cal.), and then collected bacteria were washed by passing through two 7 ml portions of filter-sterilized ASW. Three replicates were then treated as were the attached cells (see above).

Samples of attached, free-living, and detached cells were counted in polyethylene vials on a Beckman Liquid Scintillation System LS-7000 to ±2 sigma counting error or for 20 min. The counting efficiency of each sample was determined from the H-Number, an external standard type of quench monitoring [24].

Evaluation of Cell Numbers

The attached populations were prepared for microscopy by fixing the surfaces (3 remaining replicates for each incubation solution) onto glass microscope slides with double-sided adhesive tape. Five milliliter portions of suspensions of free-living and detached populations (3 replicates from each incubation solution) were filtered onto polycarbonate filters (0.2 μm porosity), which had been stained with Irgalan Black (Union Color and Chemical, Boston, Mass.) to prevent autofluorescence. The filters were then washed by passing through two 7 ml portions of ASW, air-dried, and mounted onto slides by dipping the slides in 1% (w/v) gelatin in distilled water (45°C) and placing each filter on a wet slide. Mounted samples were stained for 5 min with acridine orange (0.01% (w/v) in 6.6 mM phosphate buffer, pH 6.7) and rinsed with distilled water. Microscopic observations ensured that insignificant numbers of cells were removed from the filters during the staining procedure.

After air-drying, the specimens were examined with blue-incident light excitation under a Zeiss Standard 18 microscope using Cargille Type A immersion oil (McCrone Research Associates, London). The mean number of cells 100 μm^{-2} observed by epifluorescence (in 100 100-μm^2 areas or until at least 200 bacteria were counted for each slide) was calculated for 3 replicate samples of attached, detached, and free-living cells from each incubation solution. The total number of cells in each incubation solution was calculated for the total substratum surface area (attached cells) or volume (detached and free-living cells).

Calculation of Uptake Kinetics

The velocity of assimilation and respiration was calculated, with allowances made for unlabeled substrate carrier, nonbiological ^{14}C-CO_2 release, and ^{14}C-substrate adsorption onto the surfaces. The velocity of assimilation of attached, detached, and free-living cells was divided by the number of cells in each population to obtain the mean velocity of assimilation per cell. The total velocity of uptake per cell was calculated by dividing the sum of the velocities of assimilation and respiration by the total number of attached and detached (surface-associated) or free-living cells in the sample. V_{max} per cell and K were obtained from a modified Lineweaver-Burke plot of total uptake per cell (substrate concentration/velocity vs. substrate concentration) [38]. The utilization of amino acids by surface-associated and free-living cells was compared by calculating a carbon assimilation quotient (CAQ), that is, the amount of carbon assimilated divided by the total carbon uptake, expressed as a percentage [37].

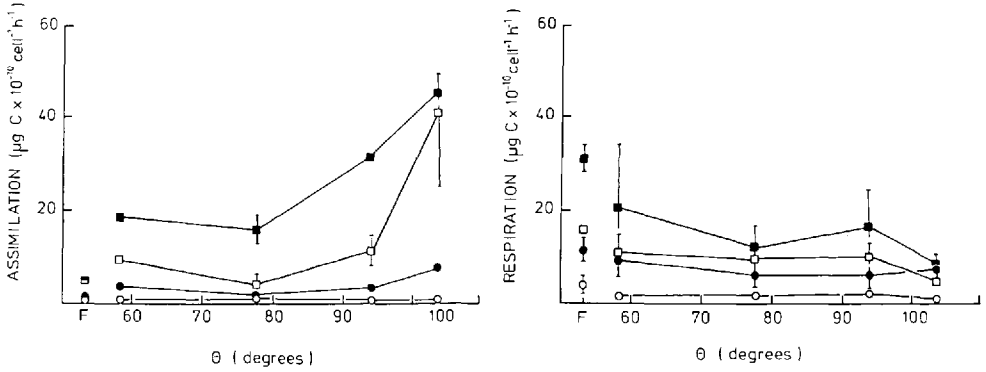

Fig. 1. Relationship between velocity of leucine assimilation by surface-associated bacteria and substratum θ_A. F is assimilation by free-living bacteria. Leucine concentrations are 10 (○), 100 (●), 500 (□), and 1,000 (■) μg C l ASW^{-1}.

Fig. 2. Respiration of leucine by surface-associated bacteria and substratum θ_A. F is respiration by free-living bacteria. Leucine concentrations are 10 (○), 100 (●), 500 (□), and 1,000 (■) μg C l ASW^{-1}.

Results

Relationship Between Substratum θ_A and Assimilation and Respiration of Leucine by Surface-Associated and Free-Living Cells

The rates of assimilation by surface-associated cells were generally independent of substratum θ_A, except that assimilation tended to increase somewhat with increase in θ_A at the 2 highest leucine concentrations (Fig. 1). Also, at these concentrations the rates of assimilation of surface-associated cells were generally higher than those of free-living cells (Fig. 1).

It was impossible to measure respiration of attached and detached cells separately, as CO_2 from both populations was collected on the same wick. Thus, all respiration measurements deal with surface-associated (attached and detached) and free-living bacteria. The mean velocity of leucine respiration by surface-associated bacteria did not differ with substratum θ_A (Fig. 2). Also, respiration of surface-associated cells was similar to or lower than that of corresponding free-living cells (Fig. 2).

The velocity of total leucine uptake (assimilated plus respired substrate) did not vary markedly with substratum θ_A (Fig. 3). An exception was cells associated with PTFE and incubated with 500–1,000 μg C l^{-1} leucine. These generally had higher uptake levels than corresponding bacteria associated with all other surfaces. Total mean uptake by surface-associated bacteria was similar to that of free-living bacteria (Fig. 3).

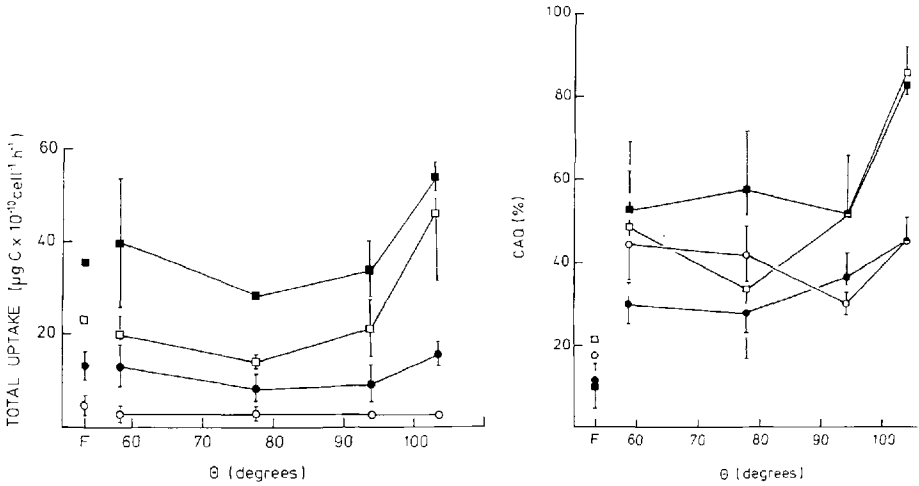

Fig. 3. Total uptake (assimilation and respiration) of leucine by surface-associated bacteria. **F** is uptake by free-living bacteria. Leucine concentrations are 10 (○), 100 (●), 500 (□), and 1,000 (■) μg C l ASW^{-1}.

Fig. 4. Carbon assimilation quotients (CAQs) of surface-associated bacteria. **F** is CAQs for free-living bacteria. Leucine concentrations are 10 (○), 100 (●), 500 (□), and 1,000 (■) μg C l ASW^{-1}.

Table 1. Kinetic parameters of leucine uptake for attached and unattached *Pseudomonas*

Population	Surface	K^b (μg C l^{-1})	$V_{max}{}^b$ (μg C cell^{-1} h^{-1} 10^{-10})	P[a]
Surface-associated	T	298	40.7	0.05
Surface-associated	PVDF	336	32.8	0.05
Surface-associated	PE	330	39.8	0.05
Surface-associated	PTFE	345	72.0	0.005
Free-living		183	40.4	0.005

[a] Level of P at which r^2 is significant for linear regression for modified Lineweaver-Burke plot.
[b] Uptake kinetics calculated from total velocity of uptake cell^{-1} at 10, 100, 500, 1,000 μg C l^{-1} leucine for 2 experiments.

Relationship Between θ_A and CAQs of Bacteria Incubated with Leucine

With most surfaces, substratum θ_A had little effect on CAQ. However, at 500–1,000 μg C l^{-1}, the CAQs for cells associated with PTFE were considerably higher than those for cells associated with the other surfaces (Fig. 4). The means of CAQs of surface-associated bacteria were generally considerably higher than those for free-living cells (Fig. 4).

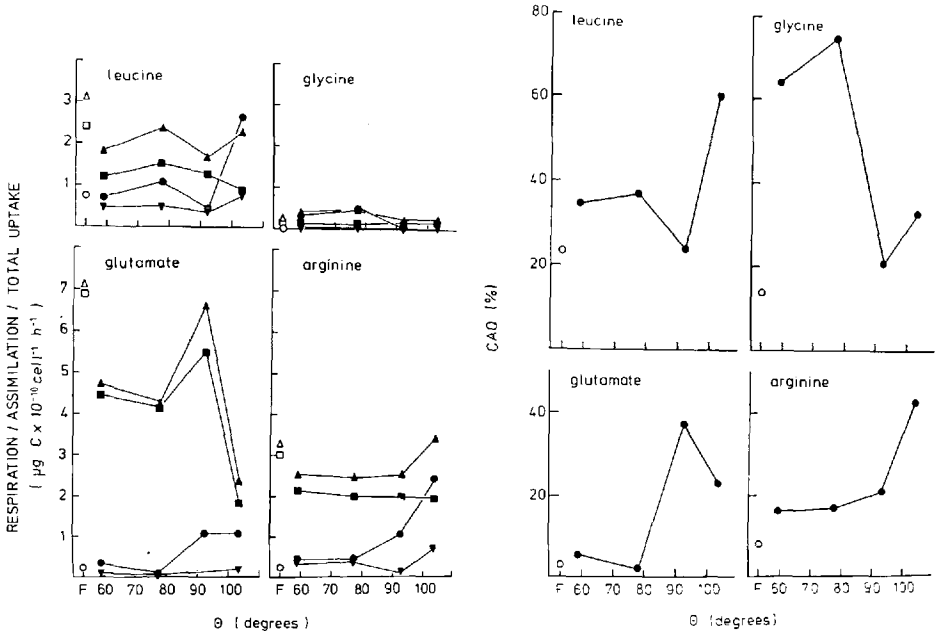

Fig. 5. Effect of amino acid concentration and substratum contact angle on assimilation, respiration or total uptake of amino acids. Symbols: Assimilation by attached (●) or detached (▼) bacteria; respiration (■) or total uptake (▲) of amino acids by surface-associated bacteria; assimilation (○), respiration (□), or total uptake (△) by free-living bacteria (F).

Fig. 6. Carbon assimilation quotients (CAQs) of surface-associated bacteria for 4 amino acids. F is the CAQ for free-living bacteria. (Based on data in Fig. 5).

Relationship Between θ_A and Transport Kinetics of Leucine

The total velocity of uptake of leucine per cell over the range 10–1,000 µg C l^{-1} leucine indicated saturation kinetics, and when transformed to a modified Lineweaver-Burke plot, showed a high degree of linearity (Table 1). The values of K for surface-associated bacteria on the different substrata were similar and considerably higher than those for free-living organisms. V_{max} per cell for bacteria associated with PTFE was considerably higher than values for organisms associated with the other surfaces or for free-living cells.

Relationship Between θ_A and Velocity of Bacterial Assimilation and Respiration of Leucine, Glycine, Glutamate, and Arginine

There was no general relationship between amino acid utilization and substratum θ_A for the 4 amino acids (Fig. 5). The rate of assimilation by attached bacteria was similar to that of detached or free-living cells, except bacteria attached to PTFE (leucine, glutamate, arginine) or PE (glutamate, arginine) had

higher assimilation rates. The substratum θ_A had little effect on respiration of attached and detached bacteria, except for cells attached to PE (glutamate) and PTFE (leucine, glutamate). Respiration of free-living bacteria was higher than or similar to (glycine) that of surface-associated cells. Total uptake of the different amino acids was not greatly affected by substratum θ_A, except with glutamate, and much smaller differences occurred with leucine and arginine. Total uptake by free-living cells was higher than, or less often, similar to, that of surface-associated cells.

The CAQs of surface-associated bacteria (Fig. 6) varied with the substratum θ_A and amino acid and followed a similar pattern to total uptake, except with glycine. The CAQs for surface-associated cells was greater than, or less often, similar to (PE and leucine, T or PVDF and glutamate) that for free-living cells.

Discussion

If the proximity of a solid surface in some way affects the activity of attached bacteria, then there could be differences in the extent of this "surface effect" with substrata of different physicochemical properties. One way of characterizing these different properties is with respect to the types of interactions (e.g., van der Waals dispersion forces, electrostatic and polar interactions and hydrogen bonding) in which the surface can participate. A parameter used to roughly evaluate the potential interaction of a surface is the advancing contact angle of water (θ_A), as an increase in the number or extent of interactions between a substratum and water is reflected by a decrease in θ_A, or in hydrophobicity.

Some studies have shown that attachment can increase the metabolic efficiency of bacteria and yeasts. For example, the molar growth yield for adsorbed *Escherichia coli* was higher than that for equivalent free-living cells [20]. Adsorption or covalent binding of *Saccharomyces carlsbergensis* altered its metabolic efficiency, as measured by an increase in the yield of glucose converted to ethanol and a decrease in the yield converted to CO_2 [31]. Also, the assimilation and respiration of amino acids by this marine pseudomonad, evaluated by microautoradiography [4, 8] and tetrazolium staining [4], have suggested that the physicochemical properties of solid surfaces can affect the activity of attached bacteria. A frequently accepted reason given for such enhanced activity at surfaces is that nutrients are adsorbed and concentrated at the solid/liquid interface [29]. Thus, some indication of the influence of substratum properties on substrate adsorption and concentration may be obtained by comparing the uptake kinetics of free-living cells and cells associated with a variety of surfaces.

An alternative suggestion for enhanced activity at surfaces is that attachment may facilitate active uptake of ions and substrates and excretion of ions and metabolites by influencing chemiosmotic processes [7] or by altering the cell's surface potential [39]. If attached bacteria thus have a lower energy requirement for nutrient assimilation, as compared with free-living cells, this may be reflected in the proportion of substrate taken up which is incorporated in cell material for growth, that is, the efficiency of substrate utilization.

In this investigation, assimilation and respiration of ^{14}C-amino acids were

measured to determine the effect of the substratum on uptake kinetics and efficiency of substrate utilization. For comparison of the uptake kinetics of surface-associated and free-living cells, the velocity of assimilation, respiration, and total uptake was calculated on a per cell basis over the substrate range 10–1,000 μg C l^{-1}. Supplementary observations using microautoradiography and tetrazolium staining demonstrated that given sufficient substrate and time, all cells were capable of amino acid assimilation and respiration, although individual cell rates varied [4].

For a valid comparison of assimilation of radiolabeled amino acids by attached cells, the cells must either be removed from the surfaces before scintillation counting, or individual corrections must be made for different levels of absorption of β-emissions by the various surfaces [33]. In this investigation, bacteria were removed from the surfaces by digestion with Luma-solv™ (see Methods).

The total velocity of uptake per cell for both surface-associated and free-living bacteria showed saturation kinetics, as demonstrated by the linearity of the data when transformed to a modified Lineweaver-Burke plot (Table 1). Values for V_{max} $cell^{-1}$ and K were obtained from this plot, and CAQs were calculated (see Methods). Surface-associated bacteria had higher K values than free-living cells (Table 1), and there are 2 possible reasons for these different values.

First, the differences in K may have been apparent, and not indicative of a real change in cell affinity for substrate. The accessibility of substrate at the solid/liquid interface may have been lower than that in the bulk phase, thereby producing higher apparent K values for surface-associated cells. Such lower accessibility of substrate may have been caused by uptake of amino acids by attached cells, producing a concentration gradient and diffusion limitation across the $\simeq$10–100 μm thick stationary layer [22] situated at the interface. Similar explanations have been proposed for the comparatively high values of apparent K reported for adsorbed bacteria [20] and immobilized enzymes [15]. Also, apparent K values for surface-associated cells may have been increased if the surfaces had adsorbed unknown contaminating substrate (Su), carried over from the original suspending medium, in which case the observed value of K would be equivalent to K + Su. It is also possible that the measured assimilation values for free-living cells may have been spuriously low if a larger proportion of assimilated material leached out of the cells after acidification, as compared with attached cells.

Secondly, the higher K values for surface-associated cells could have reflected a real difference in assimilation ability. The transport processes of bacteria could have been affected by conditions at the interface. Alternatively, attachment may have physically reduced the number of sites available for uptake [19], although a decrease in V_{max} could be expected to accompany the reduction in uptake sites. As attachment to substrata with a range of physicochemical properties increased K values to a similar extent, the increased K values for surface-associated cells were probably due to a physical effect at the surface boundary layer, for example, diffusion limitation, and not related directly to substratum properties.

V_{max} of bacteria associated with PTFE was considerably higher than that for

other surface-associated or free-living cells. The reason for this is not clear, but at 500 and 1,000 μg C l^{-1} leucine, PTFE-associated bacteria had comparatively high assimilation rates with little increase in respiration rate. It is possible that properties of PTFE in some way facilitated rapid uptake at the comparatively high leucine concentrations. Although total uptake of leucine by surface-associated and free-living cells was somewhat similar, surface-associated bacteria generally assimilated proportionally more (Fig. 1) and respired less (Fig. 2) than corresponding free-living cells, thus producing considerable differences in CAQ values (Fig. 4).

In this investigation, the relationship between assimilation or respiration and substratum θ_A differed somewhat from that found in a microscopic study of activity [4] using the same organism and similar incubation conditions. For example, in this previous study, respiration of leucine was comparatively high for cells attached to T, and assimilation of leucine, glutamate, or arginine by cells attached to PTFE was considerably lower. These inconsistencies in data from the 2 investigations may have been due to the different rationales behind the 2 methods in that this study measured the mean metabolic activities of all the cells in the attached, detached, or free-living populations, whereas the microscopic evaluations were essentially qualitative and evaluated only the proportion of each population that reached a detectable level of activity after a given incubation time. Moreover, in this study it was not possible to measure amino acid respiration separately for attached and detached cells, as was done in the microscopic study. Thus, although the 2 studies evaluate types of activity of attached and free-living cells, the various differences in the methods used make it impossible to compare the results directly.

When incubated with low concentrations (10 μg C l^{-1}) of a range of amino acids, assimilation of attached cells was generally higher than, or similar to, that of free-living cells, depending upon the amino acid and substratum. This, as well as the lack of relationship between amino acid net charge and bacterial utilization, was also found in the microautoradiography study [4]. Respiration of attached cells was lower than or similar to (glycine) that of free-living cells, and the extent of this difference in respiration was dependent on the substratum. Consequently, the CAQs of surface-associated bacteria were also dependent upon substrate and substratum θ_A and were generally higher than the CAQs of free-living cells. These differences in CAQ may be due to (1) substrata facilitating active uptake of amino acids, thereby reducing the energy requirement of attached cells; (2) differences in the energy requirement for maintenance, biosynthesis, endogeneous respiration, or motility; (3) attachment facilitating increased energy generation by chemiosmosis [7]; (4) selective attachment of the more metabolically efficient cells; or (5) adsorption of radiolabeled metabolites onto the substrata. Not enough is known about the uptake mechanisms or bioenergetics of this organism to evaluate the importance of 1, 2, or 3. Microautoradiography evaluations of tritiated amino acid assimilation [4] have shown that more active bacteria may preferentially attach, and there may be subsequent detachment of bacteria with lower assimilation rates, as in reason 4. The Microautoradiography study also indicated that some metabolites adsorb onto the substrata (reason 5), but adsorption levels were low and not

related to substratum θ_A in the same way as was CAQ in this study. Thus, metabolite adsorption was probably not an important factor.

Acknowledgments. We thank L. Richardson for excellent technical assistance. The work was supported by a Natural Environment Research Council Research Grant GR3/3561.

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