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Kinetics of glucose and amino acid uptake by attached and free-living marine bacteria in oligotrophic waters

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Abstract Kinetics of glucose and amino acid uptake by attached and free-living bacteria were compared in the upper 70 m of the oligotrophic north-western Mediterranean Sea. Potential uptake rates of amino acids were higher than those of glucose in all the samples analysed. Cell-specific potential uptake rates of attached bacteria were up to two orders of magnitude higher than those of total bacteria, both for amino acids and glucose (0.72–153 amol amino acids cell^{-1} h^{-1} and 0.05–58.42 amol glucose cell^{-1} h^{-1} for attached bacteria and 0.34–1.37 amol amino acids cell^{-1} h^{-1} and 0.07–0.22 amol glucose cell^{-1} h^{-1} for total bacteria). The apparent K_m values were also higher in attached bacteria than in total bacteria, both for amino acids and glucose. These results would reflect the presence of different uptake systems in attached and free-living bacteria, which is in accordance with the different nutrient characteristics of their microenvironments, ambient water and particles. Attached bacteria show transport systems with low affinity, which characterise a bacterial community adapted to high concentration of substrates.

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Introduction

Aquatic free-living and attached bacteria have been comparatively characterised in terms of size and heterotrophic activity (Unanue et al. 1992, 1998; Turley and Mackie 1994; Martínez et al. 1996; Agis et al. 1998; Crump et al. 1998; Turley and Stutt 2000), growth rate (Iriberri et al. 1990; Simon et al. 1990; Unanue et al. 1992) and taxonomic composition (DeLong et al. 1993; Bidle and Fletcher 1995; Alfreider et al. 1996; Acinas et al. 1999; Crump et al. 1999). Although there may be an interchange of members between both communities, several studies have found marked taxonomic and physiological differences between them. Certain taxonomic groups are selected in the particles (DeLong et al. 1993; Acinas et al. 1999), and attached bacteria are frequently bigger and more active than free-living ones (Iriberri et al. 1987; Simon et al. 1990; Turley and Stutt 2000). Physical and chemical properties of their microenvironments are extremely different, and therefore it seems consistent to assume that their enzymes must be adapted to work efficiently in different conditions.

Although there are numerous studies comparing different characteristics of attached and free-living bacteria, there are few studies devoted to the kinetic parameters of their uptake systems (Harms and Zehnder 1994). When estimating potential uptake rates of attached and free-living bacteria it is usual to perform some previous kinetic studies to determine the saturating concentrations, but unfortunately, the kinetic parameters are often not shown. Moreover, most studies of attached and free-living bacteria have been carried out in freshwater or coastal waters, and few data exist for oligotrophic waters of open seas, where the activities are very low and sometimes undetectable.

The aim of the present study was to compare the kinetics of glucose and amino acid uptake of the attached and free-living bacterial communities, in oligotrophic waters of the Mediterranean Sea. Glucose and amino acids are considered the representative low-

molecular-weight compounds of the two main fractions of organic matter in the sea, carbohydrates and proteins (Münster and Chróst 1990; Rich et al. 1996), and most bacteria are able to transport and metabolise them. The uptake of glucose and amino acids was analysed by estimating the kinetic parameters V_{max} and K_m that characterise the transport systems of these substrates.

Materials and methods

Sampling

Samples were collected in the north-western basin of the Mediterranean Sea during two cruises; Picnic (March–April 1995) and Euromarge (June–July 1995). During the Picnic cruise one station was sampled off Nice, station B at 15 m depth. During the Euromarge cruise five stations were sampled: B2 and B5 in the Banyuls transect, S5 off the Balearic Islands, and M1 and M3 in the Marseille transect (Fig. 1). At the Euromarge stations samples were taken at 40–70 m depth, where the fluorescence profile showed a maximum value (Aquatracka III fluorometer, Chelsea Instrument).

Seawater samples were collected with 12-l Niskin bottles. We assumed that these samples contain the total bacteria, although most of these will be free-living bacteria, as large particles may be destroyed during sampling due to their fragile structure. In this paper we will refer to analysis of bacterial variables in seawater sampled by Niskin as the total bacterial population. Particulate material was collected without disruption following two sampling methods. On the Euromarge cruise particulate material was collected by using a programmed detritus sampler (PDS) (Gorsky et al. 1996), a cable-operated opening–closing sampler which is designed to sample aggregates intact. It consists of a Plexiglas cylinder of 16.5 cm internal diameter with rotating waterproof taps at each end and an electronic control unit. The rotating motor and the electronic control were constructed by Technicap S.A., France. The upper tap can be separately opened in the laboratory, and the particles can be individually sampled or gently concentrated for analyses. The 7.5 l of seawater and particles sampled by the PDS was gravity filtered through a 10 μm pore-size, 147 mm diameter Nuclepore filter. The particles retained by the filter were rinsed off the filter with 100–200 ml of 0.2 μm pore-size filtered seawater, placed in a polypropylene bottle and stored in the dark at in situ temperature until subsampling for enumeration of bacteria and estimation of glucose and amino acid uptake rates. During the Picnic cruise discrete particles were collected by SCUBA divers. Once in the laboratory the particles collected by divers were diluted with 0.2 μm filtered seawater to the final volume of 100–200 ml required to perform the kinetic experiments and the bacterial counts.

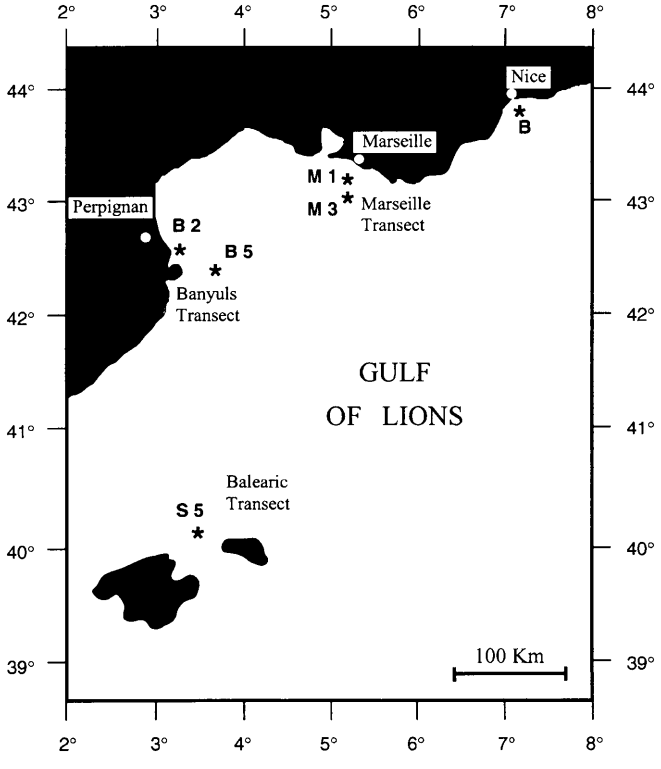

Fig. 1 Location of the sampling stations in NW Mediterranean

The in situ abundance of particles > 50 μm was determined in the Euromarge cruise by the underwater video profiler (UPV, model 2). This consists of a Hi8 Sony video camera recorder, an electronic control unit and data logger, four 24 V batteries, a light source with a collimated light field of 19.2 × 14.3 × 1.5 cm in front of the camera, and a Seabird SBE-19 CTD. This system can record one image every 4 cm, and images are processed by a system composed of a real time digitizer and software developed in collaboration with the society EFIX Informatique (Gorsky et al. 1992). The analysis of the images allows for the determination of the number and volume of particles. The degree of bacterial colonisation was estimated by dividing the abundance of attached bacteria by the volume of particulate material per litre of water as recorded by the UPV.

Bacterial counts

Attached and total bacteria were enumerated by a direct count method using epifluorescence microscopy (Hobbie et al. 1977; Turley and Hughes 1992) and the DAPI fluorochrome (Porter and Feig 1980). After preserving the samples in 0.2 μm pore-size filtered 2% glutaraldehyde (final volume) both types of samples, particles and water, were sonicated on ice for 30–120 s on a 50% on–off cycle with a ultrasonicator, which ensured the dispersion of cells without disruption. An appropriate volume (to ensure 20–30 cells field^{-1}) of sample was stained with 100 μl (for seawater) or 200 μl (for particles) of 0.2 μm filtered DAPI solution (1 mg ml^{-1} 2% glutaraldehyde) for 10 min before filtering onto a black 0.2 μm pore-size, 25 mm diameter Nuclepore filter. The filter was mounted on a slide with non-fluorescent immersion oil and a coverslip and stored frozen until enumeration on land.

Bacterial uptake of low-molecular-weight compounds

Bacterial uptake rates were determined by using D-[U-^{14}C]glucose (> 230 mCi mmol^{-1}, Amersham) and [U-^{14}C]protein hydrolysate (> 50 mCi milliatom^{-1} C, Amersham) as substrates. Labelled compounds were added to triplicate or duplicate subsamples (100 ml for seawater and 5 ml for particles) at different concentrations: 0.2, 2, 20, 200, 1000 and 2500 nM for seawater samples and 2, 20, 100, 500, 1000 and 3000 nM for particles.

Each bottle was sealed with a cap assemblage (rubber cap with a glass cup containing a Whatman N1 paper). Subsamples were incubated in the dark for 10–12 h at in situ temperature. After incubation, H_2SO_4 (final concentration, 0.02 N) was added through the cap in order to stop the incorporation, and phenethylamine (0.2 ml) was injected into the glass cup to trap the respired CO_2 (Hobbie and Crawford 1969). After 12 h, the papers impregnated in phenethylamine were placed in scintillation vials and radioassayed by liquid scintillation counting. The subsamples were filtered through 0.2 μm pore-size filters (Millipore MF), applying a vacuum pressure of 150 mmHg. The filters were rinsed three times with 10 ml of distilled water, placed in scintillation vials and radioassayed by liquid scintillation counting. These measurements represented the assimilated ^{14}C-substrate. Controls for each

substrate concentration were processed as above, but formaldehyde (final conc. 2% v/v) was added 1 h before the addition of the radiolabelled substrates. Total uptake rates for each substrate were obtained from the sum of the assimilation rate plus the respiration rate.

Kinetic parameters were estimated fitting the uptake rates to a Michaelis–Menten curve, $V = V_{max} \times S/(K'_m + S)$, by using a non-linear regression program, where V is the rate of the reaction, V_{max} is the maximum velocity of the reaction obtained at saturating concentration of the substrate, S is the concentration of substrate added, and K'_m (apparent K_m) is the concentration of substrate needed to obtain half V_{max}. K'_m represents $K_m + S_n$; K_m is the affinity constant such that a lower value for K_m indicates a higher affinity of the enzyme for the substrate; S_n is the concentration of natural substrate in the system. The study of the uptake kinetics by the incorporation of radiolabelled substrates has a major methodological problem, i.e. the presence of an unknown amount of nonlabelled natural substrate. Since we did not measure the value of S_n, the real value of K_m cannot be determined. Consequently K'_m is considered to be the maximum estimation of K_m, although this assumption will lead to a significant overestimation when the natural levels of substrate are close to the real affinity constant.

Average cell-specific potential uptake rates (V_{max} cell^{-1}) of attached and total bacteria were estimated by dividing V_{max} by the bacterial abundance; this assumes uptake by all cells.

Results and discussion

The objective of the present study was to compare attached and free-living bacteria with regard to the kinetic parameters for the uptake of glucose and amino acids. Average cell-specific potential uptake rates (V_{max} cell^{-1}) of amino acids were generally higher than those of glucose, both for total bacteria and for attached bacteria (Fig. 2; Table 1). In seawater samples V_{max} cell^{-1} for amino acids was four to eight times higher than that of glucose, while in the particles the ratio V_{max} amino acids/V_{max} glucose was very variable, ranging from 1 to 24, probably due to the different chemical composition of the particles from one sample to another. In view of these results, attached and free-living bacteria seem to show a preference for amino acids, which provide both carbon and nitrogen for bacterial growth. Moreover, the incorporation of amino acids directly into proteins will result in substantial savings in the energy cost of the synthesis of proteins.

V_{max} cell^{-1} of total bacteria were similar from one station to another in spite of the distance between stations distributed in different areas of the north-western Mediterranean Sea. V_{max} cell^{-1} of total bacteria for amino acids ranged from 0.34 to 1.37 amol cell^{-1} h^{-1} (variation coefficient = 67%), and V_{max} cell^{-1} of total bacteria for glucose ranged from 0.07 to 0.22 amol cell^{-1} h^{-1} (variation coefficient = 57%). In the case of attached bacteria the variation in V_{max} cell^{-1} was very much higher, ranging for amino acids from 0.72 to 153 amol cell^{-1} h^{-1} (variation coefficient = 126%) and for glucose from 0.05 to 58.4 amol cell^{-1} h^{-1} (variation coefficient = 109%). The narrow range of V_{max} cell^{-1} for total bacteria, compared to the wide range (three orders of magnitude) for attached bacteria, shows that nutritional conditions in the liquid phase of the oligotrophic waters of the north-western Mediterranean Sea are not

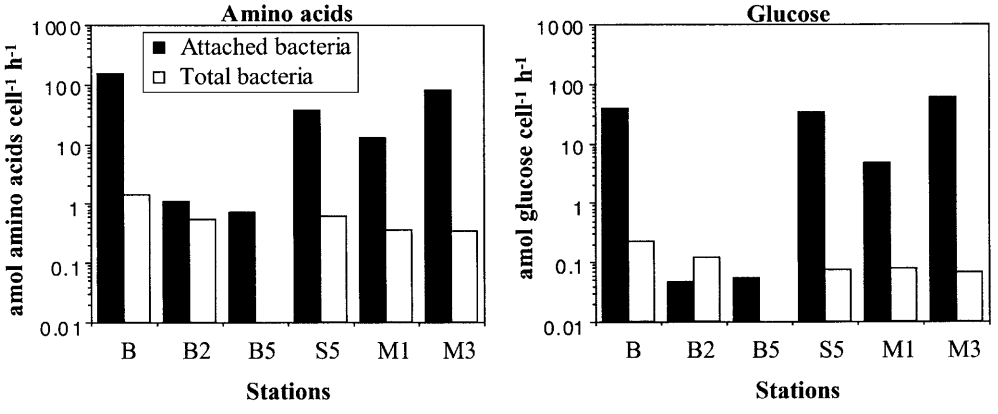

Fig. 2 Cell-specific potential uptakes rates (V_{max} cell^{-1}) of attached and total bacteria. Uptake rates of total bacteria at station B5 were not measured

Table 1 Abundance and cell-specific potential uptake rates (V_{max} cell^{-1}) of attached and total bacteria (Euromarge cruise). Uptake rates of total bacteria at station B5 were not measured

Station	Depth (m)	Attached bacteria			Total bacteria		
		Abundance (10^7 cell l^{-1})	Amino acid uptake V_{max} cell^{-1} (amol cell^{-1} h^{-1})	Glucose uptake V_{max} cell^{-1} (amol cell^{-1} h^{-1})	Abundance (10^7 cell l^{-1})	Amino acid uptake V_{max} cell^{-1} (amol cell^{-1} h^{-1})	Glucose uptake V_{max} cell (amol cell^{-1} h^{-1})
B2	40	3.6	1.09	0.05	129.5	0.53	0.12
B5	40	1.5	0.72	0.06	68.7	–	–
S5	70	0.5	36.39	32.31	40.6	0.59	0.07
M1	50	0.6	12.71	4.60	71.2	0.35	0.08
M3	70	0.8	80.51	58.42	80.6	0.34	0.07

highly variable. In contrast, the particles seem to offer very different conditions from one sample to another, probably depending on their nature, age, chemical composition and degree of bacterial colonisation.

As can be deduced from Fig. 2, attached bacteria were much more active than free-living bacteria in the uptake of low-molecular-weight compounds like glucose and amino acids. The potential uptake rates of attached bacteria were up to two orders of magnitude higher than those of total bacteria. The only exception was station B2 (Fig. 2), where the abundance of attached bacteria was very high although their potential uptake rates were very low compared to other samples. Very low potential uptake rates of attached bacteria were also found at station B5. The biggest difference between attached and total bacteria was found at station M3, an oligotrophic station (Yoro et al. 1997) where attached bacteria can take advantage of the accumulation of organic matter in the particulate material (Paerl 1985). Several studies have reported that attached bacteria are bigger than their free-living counterparts (Iriberri et al. 1987; Simon et al. 1990; Kirchman 1993), and consequently they have a larger surface to accommodate more uptake systems in their membranes. Unfortunately, the bacterial biovolume was not measured in this study, but the larger surface of attached bacteria could not fully explain the great differences in cell-specific potential uptake rates.

The stations B2 and B5 contrasted with the other stations (M1, M3, S5) with regard to the abundance and the activity of attached bacteria (Table 1). Higher abundance and lower potential uptake rates of attached bacteria were found at stations B2 and B5 compared to the other three stations. M3 and S5 are characterised by more pronounced oligotrophy than B2 and B5, which receive nutrient inputs from coastal and/or bottom influence (Conan et al. 1999). M1 is a coastal and shallow station which represents an intermediate situation. Particles at station B5 were larger and showed a lower degree of colonisation (cells μl^{-1} particles) and activity per cell than those at the stations M1, M3 and S5 (Table 2). These differences could be attributed to the age of the particles as was proposed by Turley and Stutt (2000) when analysing the variation of the cell-specific bacterial leucine incorporation of attached bacteria with depth during the Euromarge cruise. Following the hypothesis of these authors, particles from stations B2 and B5 would be newly formed, and therefore the hydrolytic enzymes of attached bacteria may not have been induced long enough to stimulate bacterial growth on the particles. Particles collected at stations M1, M3 and S5 could have been partially solubilised to utilisable dissolved organic matter, and consequently they were smaller and were colonised by more active and numerous bacterial communities than those sampled at stations B2 and B5. Moreover, the coastal and bottom influence, with inputs of recalcitrant or mineral material, could increase the size of the particles at these stations, but reduce the quality of the particles for bacterial growth.

With regard to the second kinetic parameter, the K'_m values of total bacteria for amino acid uptake systems ranged from 0.029 to 0.329 μM (variation coefficient = 79%) and for glucose from 0.043 to 0.220 μM (variation coefficient = 59%). In the case of attached bacteria the K_m values for amino acids ranged from 0.335 to 0.528 μM (variation coefficient = 18%) and for glucose from 0.025 to 2.53 μM (variation coefficient = 118%). The low variability of the K'_m values of attached bacteria for amino acid uptake systems is striking compared to those for glucose uptake (Fig. 3). If we assume that the K'_m value represents the concentration of substrate available to bacteria (Jørgensen and Søndergaard 1984; Fuhrman and Ferguson 1986), these

Table 2 Degree of bacterial colonisation and mean volume of particles (Euromarge cruise). Volume of particles at station B2 was not measured

Station	Degree of colonisation (10^6 cells μl^{-1} particles)	Volume of particles (μl)
B2	–	–
B5	0.461	0.203
S5	3.511	0.039
M1	4.035	0.018
M3	5.779	0.025

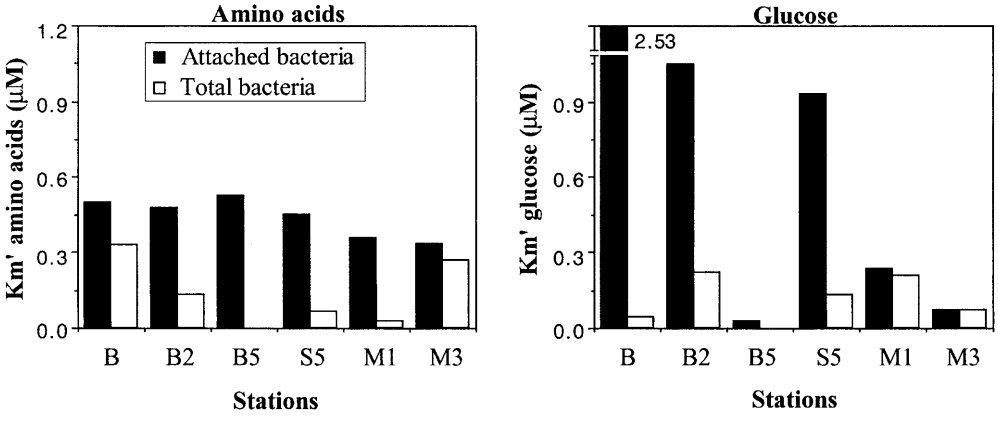

Fig. 3 K'_m (apparent K_m) values for amino acid and glucose uptake in attached and total bacteria. K'_m values of total bacteria at station B5 were not measured

results suggest that the concentration of glucose in particles is more variable than that of amino acids.

The $K'_m(K_m + S_n)$ values were higher in the particulate material than in the bulk seawater (Fig. 3). Since K_m and S_n values are unknown, these results may be due to higher concentrations of natural substrates in particles than in the ambient water, or higher values of the affinity constant for attached bacteria than for free-living bacteria, or both. High K_m values for attached bacteria would reflect the presence of low affinity uptake systems for amino acids and glucose. Particles are enriched in organic matter, and, consequently, bacteria associated with the particulate material should be adapted to high concentrations of substrates, and express enzymes with low affinity for the substrate when compared to the free-living bacteria. An additional explanation for the much higher K'_m of attached cells is provided by Harms and Zehnder (1994), who showed that the diffusion of the substrate to cells located on crowded surfaces is considerably reduced and greatly increases the K'_m, which depends on the cellular density.

From the Michaelis–Menten equation the concentration of substrate needed to obtain 90% of V_{max} can be estimated: $90V_{max}/100 = V_{max} \times S/(K'_m + S)$. The solution of this equation shows that 90% of V_{max} is reached at concentrations of $9 \times K'_m$. Consequently we can estimate that saturation was reached in seawater at concentrations of substrate ranging from 0.3 to 3 μM for amino acids and from 0.4 to 2 μM for glucose, while in particulate material the saturating concentrations for amino acids ranged from 3 to 5 μM and for glucose from 0.2 to 23 μM. It is evident that if the objective of the study is to compare the potential activities of attached and free-living bacteria, the concentrations of the added substrates should be very much higher when analysing the particulate material. Otherwise the activities of attached bacteria will be substantially underestimated.

In summary, these results point out that in the oligotrophic waters of the north-western Mediterranean Sea, the two bacterial communities, bacteria attached to particulate material and free-living bacteria, show transport systems with different kinetic constants, probably as a consequence of the nutritional status of their microenvironment, particles or ambient water. Moreover, these results emphasise the relevance of the kinetic experiments by themselves to determine, not only the saturating concentration of the substrate, but also the kinetic parameters, which provide us valuable information about the nutritional conditions of the bacterial community.

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